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(54) Title: COMPOSITION AND METHOD FOR ENHANCING FIBRINOLYSIS USING ANTIBODIES TO ALPHA-2-ANTIPLASMIN (57) Abstract <p>The present invention relates to a novel alpha-2-antiplasmin-binding molecules and treatment for pulmonary embolism, myocardial infarction, thrombosis or stroke in a patient which comprises administering an alpha-2-antiplasmin-binding molecule capable of preventing inhibition of plasmin by endogenous alpha-2-antiplasmin. The invention also relates to a treatment for pulmonary embolism, myocardial infarction, thrombosis or stroke in a patient comprising coadministering an alpha-2-antiplasmin-binding molecule of the invention together with a thrombolytic agent.</p>		

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COMPOSITION AND METHOD FOR ENHANCING FIBRINOLYSIS USING ANTIBODIES TO
ALPHA-2-ANTIPLASMIN***Background of the Invention******Statement as to Rights to Inventions Made Under
Federally-Sponsored Research and Development***

5 This invention was made in part with Government support under Contract
#HL-02348 awarded by the National Institutes of Health. The Government has
certain rights in this invention.

Field of the Invention

10 The present invention relates to a composition and method of treatment
for pulmonary embolism, myocardial infarction, thrombosis, and stroke in a
patient, and more specifically to a therapy which enhances fibrinolysis comprising
administering an alpha-2-antiplasmin-binding molecule. The invention also relates
to a treatment for enhancing fibrinolysis comprising administering an alpha-2-
antiplasmin-binding molecule together with a thrombolytic agent.

15 ***Description of Background Art***

 Venous thrombosis and pulmonary embolism are major causes of
morbidity and mortality in the United States, accounting for about 270,000
hospitalizations a year (Anderson, F.A., Jr. *et al.*, *Arch. Intern. Med.* 151:933-938
(1991)). In addition, it is estimated that about 50,000-200,000 patients a year die
20 from pulmonary embolism (Lilienfeld, D.E. *et al.*, *Chest* 98:1067-1072 (1990)).
In surprising contrast with the mortality rate for myocardial infarction, the
mortality rate for pulmonary embolism (estimated at 9.2% in treated patients) has
not improved in the last 30 years (Lilienfeld, D.E. *et al.*, *Chest* 98:1067-1072
(1990); Giuntini, C. *et al.*, *Chest* 107:3S-9S (1995)). Moreover, survivors of
25 venous thromboembolism are known to be at risk for recurrent thrombosis,

postphlebitic syndrome, and pulmonary hypertension (Sutton, G.C. *et al.*, *Br. Heart J.* 39:1135-1192 (1977); Salzman, E.W. and Hirsch, J., "The Epidemiology, Pathogenesis and Natural History of Venous Thrombosis," in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, Coleman, R.W. *et al.*, eds., 3rd ed. Philadelphia, PA (1994), pp. 1275-1296).

A. *Mechanism of Clot Formation and Lysis*

Clots (or thrombi in a patient) are composed of fibrin and blood platelets in various ratios. The fundamental reaction in blood clotting involves the conversion of a soluble plasma protein (fibrinogen) into insoluble fibrin. The conversion of fibrinogen into fibrin is catalyzed by the enzyme, thrombin, which is a serine protease.

Clot lysis is mediated by plasmin. Under natural conditions, plasminogen is converted to plasmin by plasminogen activators. Natural plasmin inhibitors include α 2-antiplasmin, α 2-macroglobulin and α -1-antitrypsin, all glycoproteins. Alpha-2-antiplasmin has a much higher affinity for plasmin than α 2-macroglobulin and binds specifically to plasmin in a 1:1 ratio. The larger pool of α -macroglobulin acts as a reservoir inhibitor (Kane, K.K., *Ann. Clin. Lab. Sci.* 14:443-449 (1984)). Thus, clot lysis by the administration of t-PA is limited by the rapid and irreversible inactivation of plasmin by plasmin inhibitors.

B. *Treatment for Venous Thrombosis and Pulmonary Embolism*

Standard therapy for venous thromboembolism is heparin, which potentiates thrombin and factor Xa inhibition by antithrombin III (Goldhaber, S., *Chest* 107:45S-51S (1995)). Although heparin decreases new thrombus (clot) formation, clinical studies suggest that there is little early endogenous lysis of the large thrombi that often exist at the time of diagnosis in patients with venous

thromboembolism (Goldhaber, S.Z. *et al.*, *Lancet* 2:886-889 (1986); "The Urokinase Pulmonary Embolism Trial," *Circulation* 47:1-108 (1973); Goldhaber, S.Z. *et al.*, *Am. J. Med.* 88:235-240 (1990); Goldhaber, S.Z. *et al.*, *Lancet* 341:507-511 (1993)). Since large thrombi are associated with an increase in morbidity and mortality, several studies have examined the effects of plasminogen activators in patients with venous thromboembolism (Goldhaber, S.Z. *et al.*, *Lancet* 2:886-889 (1986); "The Urokinase Pulmonary Embolism Trial," *Circulation* 47:1-108 (1973); Goldhaber, S.Z. *et al.*, *Am. J. Med.* 88:235-240 (1990); Goldhaber, S.Z. *et al.*, *Lancet* 341:507-511 (1993)).

Compared with heparin alone, plasminogen activators cause significant increases in the lysis of venous thromboemboli, but patients are frequently left with large amounts of residual thrombi in the lungs or deep veins immediately after therapy (Goldhaber, S.Z. *et al.*, *Lancet* 2:886-889 (1986); "The Urokinase Pulmonary Embolism Trial," *Circulation* 47:1-108 (1973); Goldhaber, S.Z. *et al.*, *Am. J. Med.* 88:235-240 (1990); Goldhaber, S.Z. *et al.*, *Lancet* 341:507-511 (1993)). None of the randomized, controlled trials of patients with pulmonary embolism have demonstrated a mortality benefit from plasminogen activators, although this may well be due to the small numbers of patients enrolled in these studies. Use of plasminogen activators for myocardial infarctions has shown that 45-70% of patients with coronary thrombosis have failed to achieve full 90 minutes reperfusion with these agents.

Why venous thromboemboli resist fibrinolysis is unknown. Physical characteristics such as size, retraction, exposure to blood flow, and age may affect the lysis of these large fibrin-rich thrombi (Prewitt, R.M., *Chest* 99:157S-164S (1991)). However, it is also likely that the fibrinolytic resistance of these thrombi is regulated by specific molecular factors such as factor XIII, plasminogen activator inhibitor 1 (PAI-1), and alpha-2-antiplasmin (α 2AP) (Collen, D., *Eur. J. Biochem.* 69:209-216 (1976); Moroi, M. and Aoki, N., *J. Biol. Chem.* 251:5956-5965 (1976); Mullertz, S. and Clemmensen, I., *Biochem. J.* 159:545-553 (1976); Sakata, Y. and Aoki, N., *J. Clin. Invest.* 69:536-542 (1982); Robbie,

L.A. *et al.*, *Thromb. Haemostas.* 70:301-306 (1993); Francis, C.W. and Marder, V.J., *J. Clin. Invest.* 80:1459-1465 (1987); Jansen, J.W.C.M. *et al.*, *Thromb. Haemostas.* 57:171-175 (1987); Reed, G.L. *et al.*, *Trans. Assoc. Am. Phys.* 104:21-28 (1991); Stringer, H.A. and Pannekoek, H., *J. Biol. Chem.* 270:11205-11208 (1995); Carmeliet, P. *et al.*, *J. Clin. Invest.* 92:2756-2760 (1993); Lang, I.M. *et al.*, *Circulation* 89:2715-2721 (1994); Marsh, J.J. *et al.*, *Circulation* 90:3091-3097 (1994)).

Because α 2AP is an ultrafast covalent inhibitor of plasmin (the enzyme that degrades thrombi), α 2AP is a particularly likely cause of thrombus resistance (Collen, D., *Eur. J. Biochem.* 69:209-216 (1976); Moroi, M. and Aoki, N., *J. Biol. Chem.* 251:5956-5965 (1976); Mullertz, S. and Clemmensen, I., *Biochem. J.* 159:545-553 (1976)). Moreover, α 2AP is the only fibrinolytic inhibitor that is covalently crosslinked to the fibrin surface (Sakata, Y. and Aoki, N., *J. Clin. Invest.* 69:536-542 (1982)). This crosslinking (by activated factor XIII) concentrates α 2AP on the fibrin surface, where it inhibits the initiation of fibrinolysis (Sakata, Y. and Aoki, N., *J. Clin. Invest.* 69:536-542 (1982)). Previous *in vitro* studies have shown that clots from α 2AP-deficient patients lyse spontaneously, suggesting that α 2AP plays a critical role in thrombus resistance to endogenous plasminogen activators (Aoki, N. *et al.*, *Blood* 62:1118-1122 (1983); Miles, L.A. *et al.*, *Blood* 59:1246-1251 (1982)). These observations led to the hypothesis that α 2AP is a molecular mediator of the thrombus resistance seen in patients with pulmonary embolism. To test this hypothesis, we generated a specific inhibitor of α 2AP and used it to determine the role played by α 2AP in the regulation of lysis of experimental pulmonary emboli.

If an individual has formed a fibrin clot (thrombus) prior to the availability of medical assistance, the clot may be dissolved through the use of agents capable of lysing the fibrin thrombus, and thereby permitting blood to again flow through the affected blood vessel. Such agents include plasmin, anti-coagulants (such as, for example, heparin, hirudin and activated protein C), plasminogen activators (such as, for example, streptokinase, prourokinase, urokinase, tissue-type

plasminogen activator, staphylokinase, and vampire bat plasminogen activator), and other such agents (Ganz, W. *et al.*, *J. Amer. Coll. Cardiol.* 1:1247-1253 (1983); Rentrop, K.P. *et al.*, *Amer. J. Cardiol.* 54:29E-31E (1984); Gold, H.K. *et al.*, *Amer. J. Cardiol.* 53:122C-125C (1984)).

5 At present, treatment of pulmonary embolism, myocardial infarction, thrombosis, and stroke is partially achieved through the administration of thrombolytic agents. Use of such agents in therapy often results in incomplete lysis, and promotes the reformation of thrombi and reocclusion of the affected blood vessels. Hence, a need exists for an improvement in thrombolytic therapy
10 which enhances fibrinolysis, while minimizing fibrinogen breakdown and preventing reformation of thrombi.

C. *Alpha-2 antiplasmin Antibodies*

Alpha-2-antiplasmin (α 2AP) has three functional domains: the reactive site for plasmin, the plasmin(ogen) or LBS-binding site [complementary to the
15 LBS (lysine-binding site) of plasmin(ogen)], and the crosslinking site for fibrin. Mimuro, J. *et al.*, *Blood* 69:446-453 (1987). Mimuro *et al.* discloses antibodies to α 2AP, one of which (JPTI-1) was specific to the reactive site of α 2AP and prevented formation of α 2AP complexes, thereby inhibiting antiplasmin activity. However, Mimuro *et al.* does not teach administration of the JPTI-1 antibody to
20 enhance clot lysis. Other antibodies specific for α 2AP are taught by Plow, E.F. *et al.*, *J. Biol. Chem.* 255:2902-2906 (1980); Wimen, B. *et al.*, *Scan. J. Clin. Lab. Invest.* 43:27-33 (1983); Hattey, E. *et al.*, *Thromb. Res.* 45:485-495 (1987); Collen, U.S. Patent No. 4,346,029 (1980); and Collen, U.S. Patent No. 4,198,335 (1980).

Summary of the Invention

The present invention relates to an improved thrombolytic therapy for the treatment of pulmonary embolism, myocardial infarction, thrombosis and stroke in patients. The invention is directed to an immunologic molecule capable of binding to both (1) human and nonhuman circulating $\alpha 2$ -antiplasmins and (2) human and nonhuman fibrin crosslinked $\alpha 2$ -antiplasmins. In preferred embodiments, the immunologic molecule is a chimeric antibody, a humanized antibody, or a single chain antibody. The invention is also directed to a method for treating pulmonary embolism, myocardial infarction, thrombosis and stroke in a patient comprising administering an $\alpha 2$ -antiplasmin-binding molecule capable of binding to both (1) human and nonhuman circulating $\alpha 2$ -antiplasmins and (2) human and nonhuman fibrin crosslinked $\alpha 2$ -antiplasmins. The invention further provides a method of treatment for pulmonary embolism, myocardial infarction, thrombosis and stroke in a patient which comprises co-administrating to a patient in need of such treatment:

(a) a therapeutically effective amount of an immunologic molecule capable of binding to both (1) human and nonhuman circulating $\alpha 2$ -antiplasmins and (2) human and nonhuman fibrin crosslinked $\alpha 2$ -antiplasmins; and

(b) a therapeutically effective amount of a thrombolytic agent, wherein the immunologic molecule (a) is different from the thrombolytic agent (b), thereby treating the patient.

The invention provides a monoclonal antibody or fragment thereof wherein the monoclonal antibody is capable of binding to both (1) human and nonhuman circulating $\alpha 2$ -antiplasmins and (2) human and nonhuman fibrin crosslinked $\alpha 2$ -antiplasmins. In one embodiment, the invention is monoclonal antibody 77A3. In another embodiment, the invention is monoclonal antibody 49C9. In another embodiment, the monoclonal antibody is 70B11.

The invention also provides a method of making the monoclonal antibody comprising:

- (a) immunizing an animal with α 2-antiplasmin or fragment thereof;
- (b) fusing cells from the animal with tumor cells to make a hybridoma cell line;
- (c) cloning the hybridoma cell line;
- 5 (d) selecting for the monoclonal antibody capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins; and
- (e) obtaining the monoclonal antibody.

10 The invention provides a hybridoma cell line which produces the monoclonal antibody capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins. In one embodiment, the invention is hybridoma cell line 77A3 (ATCC Accession No. HB-12192; Deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20862 on September 20,

15 1996).

The invention is directed to a method of making the hybridoma cell line comprising:

- (a) immunizing an animal with α 2-antiplasmin or fragment thereof;
- (b) fusing the cells from the animal with tumor cells to make the
- 20 hybridoma cell line; and
- (c) obtaining the hybridoma cell line which produces the monoclonal antibody capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins.

25 The invention also provides a method for treating a number of diseases and conditions, including pulmonary embolism, myocardial infarction, thrombosis and stroke in a patient comprising administering a therapeutically effective amount of an immunologic molecule which is capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins, thereby treating the patient.

The invention further provides a method of treatment for pulmonary embolism, myocardial infarction, thrombosis or stroke in a patient which comprises co-administering to a patient in need of such treatment:

5 (a) a therapeutically effective amount of an immunologic molecule which is capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins; and

(b) a therapeutically effective amount of a thrombolytic agent, wherein the immunologic molecule (a) is different from the thrombolytic agent (b), thereby treating the patient.

10 In preferred embodiments, the thrombolytic agent is plasmin, anti-coagulant, or plasminogen activator. In one embodiment, the anti-coagulant is selected from the group consisting of heparin, hirudin and activated protein C. In another embodiment, the plasminogen activator is selected from the group consisting of staphylokinase, streptokinase, prourokinase, urokinase, tissue-type
15 plasminogen activator, and vampire bat plasminogen activator.

Other embodiments of the invention include, the immunologic molecule provided to the patient by an intravenous infusion, by an intravenously injected bolus, or with a first bolus containing the immunologic molecule (a) and a subsequently administered second bolus containing the thrombolytic agent (b).
20 Further embodiments include, the immunologic molecule (a) provided to the patient at a dose of between 3 to 300 nmole per kg of patient weight; and the thrombolytic agent (b) provided to the patient at a dose of between 0.01 to 3.0 mg per kg of patient weight.

25 The invention provides a kit useful for carrying out the method of treatment for pulmonary embolism, myocardial infarction, thrombosis or stroke in a patient, being compartmentalized in close confinement to receive two or more container means therein, which comprises:

(1) a first container containing a therapeutically effective amount of the immunologic molecule (a); and

(2) a second container containing a therapeutically effective amount of the thrombolytic agent (b), wherein the immunologic molecule (a) is different from the thrombolytic agent (b).

The invention also provides nucleic acid molecules encoding immunologic molecules capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins. Also provided are molecules comprising an amino acid sequence of the binding region of an immunologic molecule described herein.

Brief Description of the Figures

Figure 1. Comparison of binding to ^{125}I - α 2-antiplasmin of monoclonal antibodies 49C9, 70B11, 77A3, RWR and anti-digoxin (control). Wells of a microtiter plate were coated with goat antimouse antibody. The wells were incubated in duplicate with 49C9, 70B11, 77A3, RWR or a control (antidigoxin) MAb (Mudgett-Hunter, M. *et al.*, *Mol. Immunol.* 22:477-488 (1985)). After a wash, ^{125}I - α 2AP (60,000 cpm) was added for an hour. The wells were rinsed and the amount of bound ^{125}I - α 2AP was measured in a gamma counter.

Figure 2. Competition binding assays of monoclonal antibodies 49C9, 70B11, 77A3, RWR and anti-digoxin with immobilized 70B11. Competition radioimmunoassays were performed by coating wells of a microtiter plate with 25 μl of purified MAb (70B11) in duplicate (10 $\mu\text{g}/\text{ml}$) for 1 hour. The wells were washed and blocked with 1% BSA for 1 hour. After washing, 25 μl of a competitor MAb, same MAb or negative control MAb was added to different wells (50 $\mu\text{g}/\text{ml}$) followed by 25 μl of ^{125}I - α 2-antiplasmin (100,000 cpm). After 1 hour incubation, the wells were washed, cut and the radioactivity was measured in a gamma scintillation counter.

Figure 3. Comparison of amount of lysis by different monoclonal antibodies (or TBS alone) as a function of dose of urokinase. See Example 1, below, for detailed description of the method. The amount of lysis was determined by gamma counting. The percent lysis was defined at $100 \times (\text{total supernatant cpm} \div \text{total clot cpm})$.

Figure 4. Dose response studies in the absence or presence of MAb 77A3. Lysis by urokinase is increased approximately 100-fold by 77A3.

Figure 5. Reduced SDS-polyacrylamide gel electrophoresis of 77A3 purification. Ascites containing 77A3 were harvested and purified. Lane 1, protein standards with molecular mass in kDa (left); lane 2, supernatant after precipitation with 40% ammonium sulfate; lane 3, purified 77A3. The reduced 77A3 immunoglobulin consists of bands of ~50 kDa, corresponding to the heavy chain, and ~25 kDa, corresponding to the light chain.

Figure 6. Effect of 77A3 on the rate of lysis of ferret plasma clots *in vitro*. Ferret plasma clots formed with trace amounts of ^{125}I -labeled human fibrinogen were incubated with 100 μl of TBS (control) or purified MAb (25 μg , 77A3 or RWR). Clot lysis was initiated by adding 0.1 unit of rt-PA per tube. The clots were incubated at 37°C and the amount of lysis was determined by sampling for the release of radiolabeled fibrin degradation products into the supernatant as described (Reed, G.L. III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990)).

Figure 7. Effect of *in vivo* administration of MAb 77A3 on functional $\alpha 2\text{AP}$ levels in ferrets. In dose finding experiments, two anesthetized ferrets (A, B) were given 77A3 intravenously (22.5 mg/kg) and the amount of functional $\alpha 2\text{AP}$ was measured in citrated plasma samples drawn before (time 0) and 1 and

4 hours after infusion. The data represent the mean \pm SD inhibition of α 2AP in plasma samples.

5 **Figure 8.** Effect of rt-PA and α 2AP inhibition on the lysis of pulmonary emboli *in vivo*. Anesthetized ferrets were given a heparin bolus (100 U/kg) and 125 I-labeled fibrin clots were embolized into the lungs. After embolization, three groups of ferrets were given rt-PA (0, 1, or 2 mg/kg) over 2 hours intravenously (plain bars). Two other groups of ferrets also received rt-PA (1 mg/kg) and a control MAb (antidigoxin, black bar, 22.5 mg/kg) or a MAb that inhibits α 2AP (77A3, striped bar, same dose). The graph shows the amount of lysis (mean \pm SD) for each treatment group. The number of ferrets in each treatment group is shown, and the *P* values for differences between groups are indicated.

15 **Figure 9.** Residual fibrinogen levels in animals treated with heparin, rt-PA, and an α 2AP inhibitor. Blood samples were collected (on EDTA with aprotinin) from ferrets before pulmonary embolization and at the end of the experiment. Residual fibrinogen levels were measured as described (Rampling, M.W. and Gaffney, P.J., *Clin. Chim. Acta.* 67:43-52 (1976)). The graph shows the mean \pm SD percentage residual fibrinogen level for animals receiving rt-PA alone (0, 1, or 2 mg/kg; plain bars) and those receiving rt-PA and the α 2AP inhibitor (striped bar).

20 **Figure 10.** The peptide sequences of the amino terminus of purified light chains from 49C9 (SEQ ID NO:1), 70B11 (SEQ ID NO:2) and 77A3 (SEQ ID NO:3) are shown.

25 **Figure 11.** The cDNA sequence (SEQ ID NO:4) and corresponding deduced amino acid sequence of the signal peptide (amino acids -20 to -1 of SEQ ID NO:5) and light chain variable regions (amino acids 1 to 107 of SEQ ID NO:5) of 49C9 are shown.

Figure 12. The cDNA sequence (SEQ ID NO:6) and corresponding deduced amino acid sequence of the signal peptide (amino acids -20 to -1 of SEQ ID NO:7) and light chain variable regions (amino acids 1 to 107 of SEQ ID NO:7) of 70B11 are shown.

5 **Figure 13.** The cDNA sequence (SEQ ID NO:8) and corresponding deduced amino acid sequence of the signal peptide (amino acids -20 to -1 of SEQ ID NO:9) and light chain variable regions (amino acids 1 to 107 of SEQ ID NO:9) of 77A3 are shown.

10 **Figure 14.** The cDNA sequence (SEQ ID NO:10) and corresponding deduced amino acid sequence of the signal peptide (amino acids -19 to -1 of SEQ ID NO:11) and heavy chain variable regions (amino acids 1-119 of SEQ ID NO:11) of 49C9 are shown.

15 **Figure 15.** The cDNA sequence (SEQ ID NO:12) and corresponding deduced amino acid sequence of the signal peptide (amino acids -19 to -1 of SEQ ID NO:13) and heavy chain variable regions (amino acids 1-119 of SEQ ID NO:13) of 70B11 are shown.

20 **Figure 16.** The cDNA sequence (SEQ ID NO:14) and corresponding deduced amino acid sequence of the signal peptide (amino acids -19 to -1 of SEQ ID NO:15) and heavy chain variable regions (amino acids 1-119 of SEQ ID NO:15) of 77A3 are shown.

Figure 17. The cDNA sequence (SEQ ID NO:16) and corresponding amino acid sequence (SEQ ID NO:17) of humanized77A3-1 and humanized 77A3-2 light chain. Positions falling within the CDR loops are shown enclosed within the boxes with solid borders.

Figure 18. The cDNA sequence (SEQ ID NO:18) and corresponding amino acid sequence (SEQ ID NO:19) of humanized 77A3-1 heavy chain. Positions falling within the CDR loops are shown enclosed within the boxes with solid borders.

5 **Figure 19.** The cDNA sequence (SEQ ID NO:20) and corresponding amino acid sequence (SEQ ID NO:21) of humanized 77A3-2 heavy chain. Positions falling within the CDR loops are shown enclosed within the boxes with solid borders.

10 **Figure 20.** Results of murine 77A3 (X), chimeric 77A3 (●) and humanized 77A3-1 (■) in the plasmin assay with chromogenic substrate are shown.

15 **Figure 21.** The amino acid sequences of the light chains are shown: h77A3-1 and h77A3-2 (SEQ ID NO:17); m77A3 (SEQ ID NO:9); m49C9 (SEQ ID NO:5); m70B11 (SEQ ID NO:7); murine consensus (SEQ ID NO:75), which shows the consensus between m77A3, m49C9, and m70B11; 77A3/49C9 consensus (SEQ ID NO:76), which shows the consensus between 77A3 and 49C9; and all (SEQ ID NO:77), which shows the consensus between h77A3-1, h77A3-2, m77A3, m49C9, and m70B11. Positions falling within the CDR loops are shown enclosed within the boxes.

20 **Figure 22.** The amino acid sequences of the heavy chains are shown. h77A3-1 (SEQ ID NO:19); h77A3-2 (SEQ ID NO:21); m77A3 (SEQ ID NO:15); m49C9 (SEQ ID NO:11); m70B11 (SEQ ID NO:13); humanized consensus (SEQ ID NO:78), which is the consensus between h77A3-1 and h77A3-2; murine consensus (SEQ ID NO:79), which is the consensus between m77A3, m49C9, and
25 m70B11; 77A3/49C9 consensus (SEQ ID NO:80), which is the consensus between 77A3 and 49C9; and all (SEQ ID NO:81), which is the consensus

between h77A3-1, h77A3-2, m77A3, m49C9, and m70B11. Positions falling within the CDR loops are shown enclosed within the boxes.

Detailed Description of the Preferred Embodiments

Alpha-2-antiplasmin (α 2AP) is a molecular mediator of the thrombus resistance in patients with pulmonary embolism. A specific inhibitor of α 2AP is described which is used to determine the role played by α 2AP in the regulation of fibrinolysis.

A. Immunologic Molecules

In the following description, reference will be made to various methodologies well-known to those skilled in the art of immunology. Standard reference works setting forth the general principles of immunology include Klein, J., *Immunology: The Science of Cell-Noncell Discrimination*, John Wiley & Sons, New York (1982); Kennett, R. *et al.*, *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, New York (1980); Campbell, A., "Monoclonal Antibody Technology," in *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 13, Burdon, R., *et al.*, eds., Elsevier, Amsterdam (1984); and Eisen, H.N., *Microbiology*, 3rd ed., Davis, B.D., *et al.*, Harper & Row, Philadelphia (1980).

As used herein, α 2AP-binding molecule includes antibodies (polyclonal or monoclonal), as well as ligands. As used herein, an "immunologic molecule" refers to polypeptides comprising the binding region of a monoclonal antibody. Thus, monoclonal antibodies, antibody fragments, chimeric antibodies, humanized antibodies, and fusion proteins comprising antibody binding regions are "immunologic molecules". The term "antibody" (Ab) or "monoclonal antibody" (MAb) is meant to include intact molecules as well as antibody fragments (such as, for example, Fv, Fab and F(ab')₂ fragments), single chain antigen-binding

proteins, "humanized" antibodies, and chimeric antibodies which are capable of specifically binding to α 2AP. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)). Thus, these fragments are preferred.

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. As used herein, the term "hapten" is intended to refer to any molecule capable of being bound by an antibody. The term "epitope" is meant to refer to that portion of a hapten which can be recognized and bound by an antibody. A hapten or antigen may have one, or more than one epitope. An "antigen" or "immunogen" is a hapten which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. The specific reaction referred to above is meant to indicate that the hapten will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing α 2AP (or fractions, lysates, etc. thereof) can be administered to an animal in order to induce the production of sera containing polyclonal antibodies that are capable of binding α 2AP. In a preferred method, a preparation of α 2AP of the present invention is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

The antibodies of the present invention may also be prepared using phage display technology. Methods of preparing antibodies using phage display are known in the art. See, for example, U.S. Patent No. 5,565,332; Clarkson *et al.*, *Nature* 352:624-628 (1991); Huse, *Science* 246:1275-1281 (1989); Kang, *Proc. Natl. Acad. Sci. USA* 88:11120-11123 (1993); Marks, *J. Mol. Biol.* 222:581-597 (1991); and McCafferty *et al.*, *Nature* 348:552-554 (1990).

In one preferred method, the immunogenic molecules of the present invention are monoclonal antibodies (or α 2AP binding molecules). Such monoclonal antibodies can be prepared using hybridoma technology (Kohler *et al.*, *Nature* 256:495 (1975); Kohler *et al.*, *Eur. J. Immunol.* 6:511 (1976); Kohler *et al.*, *Eur. J. Immunol.* 6:292 (1976); Hammerling *et al.*, in *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., pp. 563-681 (1981)). In general, such procedures involve immunizing an animal (preferably a mouse) with the antigen or with a cell which expresses the antigen. A preferred antigen is purified α 2AP. The most preferred antigen is α 2AP fragment (fibrin binding region) obtained by trypsin digest of a plasma clot, then affinity purified with a SEPHAROSE-coupled monoclonal antibody, RWR (Reed, G.L. III *et al.*, *Trans. Assoc. Am. Phys.* 101:250-256 (1988); U.S. Patent No. 5,372,812, issued December 13, 1994). Suitable cells can be recognized by their capacity to secrete anti- α 2AP antibody. Such cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56°C), and supplemented with about 10 μ g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 μ g/ml of streptomycin. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. The method of somatic cell fusion is described in Galfre, G. and Milstein, C., *Meth. Enzymol.* 73:3-46 (1981). After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands *et al.*, *Gastroenterology* 80:225-232 (1981). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding α 2AP.

Alternatively, additional antibodies capable of binding to the α 2AP antigen may be produced in a two-step procedure through the use of anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and that, therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, α 2AP-specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an

animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the α 2AP-specific antibody can be blocked by the α 2AP antigen. Such antibodies comprise anti-idiotypic antibodies to the α 2AP-specific antibody and can be used to immunize an animal to induce formation of further α 2AP-specific antibodies.

It will be appreciated that Fab and F(ab')₂ and other fragments of the antibodies of the present invention may be used according to the methods disclosed herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). Alternatively, α 2AP-binding fragments can be produced through the application of recombinant DNA technology, through synthetic chemistry, or biotinylation.

Also intended within the scope of the present invention are humanized or chimeric antibodies, produced using genetic constructs derived from hybridoma cells producing the MAbs described above. Humanized antibodies are antibodies in which the framework or other regions of the murine Ab is replaced with the homologous regions of a nonmurine antibody. Chimeric antibodies are antibodies in which the murine constant region has been replaced with a non-murine constant region. Methods for production of chimeric antibodies are known in the art. *See*, for review: Morrison, *Science*, 229:1202-1207 (1985); Oi *et al.*, *BioTechniques* 4:214 (1986); *see also*, Cabilly *et al.*, U.S. Patent 4,816,567 (3/28/89); Taniguchi *et al.*, EP171496 (2/19/86); Morrison *et al.*, EP173494 (3/5/86); Neuberger *et al.*, WO8601533 (3/13/86); Robinson *et al.*, WO 8702671 (5/7/87); Boulianne *et al.*, *Nature* 312:643-646 (1984); and Neuberger *et al.*, *Nature* 314:268-270 (1985). Methods for production of humanized antibodies are known in the art. *See*, for example, U.S. Patent 5,585,089; Jones *et al.*, *Nature* 321:522-525 (1986); and Kettleborough *et al.*, *Protein Engineering* 4:773-783 (1991).

Also provided in the present invention are single-chain antibodies capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins. Methods of making

single chain antibodies are well known in the art. *See*, for example, U.S. Patent No. 4,946,778; U.S. Patent No. 5,260,203; U.S. Patent No. 5,091,513; and U.S. Patent No. 5,455,030, all of which are herein incorporated by reference.

Also intended within the scope of the present invention are variants of the monoclonal antibodies described above.

The present inventors have determined the nucleotide and amino acid sequence of several immunologic molecules capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins. Accordingly, the present invention provides for nucleic acid molecules comprising a nucleotide sequence encoding for an immunologic molecule of the present invention or fragment thereof.

Due to the degeneracy of the genetic code, and to the fact that the genetic code is known, all other nucleotide sequences which encode the same amino acid sequence as the nucleotides of the present invention can be determined and used in the practice of the present invention.

DNA clones containing nucleotide sequences encoding the following antibody chains were deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20862 on September 19, 1997: light chain of 77A3 (77A3 LC), ATCC Accession No. _____; light chain of 49C9 (49C9 LC), ATCC Accession No. _____; light chain of 70B11 (70B11 LC), ATCC Accession No. _____; heavy chain of 77A3 (77A3 HC), ATCC Accession No. _____; heavy chain of 49C9 (49C9 HC), ATCC Accession No. _____; and heavy chain of 70B11 (70B11 HC), ATCC Accession No. _____.

The nucleic acid molecules of the present invention include: nucleic acid molecules containing a nucleotide sequence encoding the mature light chain of 77A3 as shown in SEQ ID NO:9 or as encoded by the clone contained in the ATCC Accession No. _____; nucleic acid molecules containing a nucleotide sequence encoding the mature light chain of 49C9 as shown in SEQ ID NO:5 or as encoded by the clone contained in ATCC Accession No. _____; and nucleic acid molecules containing a nucleotide sequence encoding the mature light chain

of 70B11 as shown in SEQ ID NO:7 or as encoded by the clone contained in ATCC Accession No. _____.

Also included in the present invention are nucleic acid molecules containing a nucleotide sequence encoding an antibody heavy chain, including:
5 nucleic acid molecules containing a nucleotide sequence encoding the mature heavy chain of 77A3 as shown in SEQ ID NO:15 or as encoded by the clone contained in ATCC Accession No. _____; nucleic acid molecules containing a nucleotide sequence encoding the mature heavy chain of 49C9 as shown in SEQ ID NO:11 or as encoded by the clone contained in ATCC Accession No. _____;
10 and nucleic acid molecules containing a nucleotide sequence encoding the mature heavy chain of 70B11 as shown in SEQ ID NO:13 or as encoded by the clone contained in ATCC Accession No. _____.

Also included are nucleic acid molecules encoding humanized antibodies including: nucleic acid molecules comprising a nucleotide sequence encoding for
15 amino acid residues 1 to 107 of SEQ ID NO:17; nucleic acid molecules comprising a nucleotide sequence encoding for amino acid residues 1 to 119 of SEQ ID NO:19; and nucleic acid molecules comprising a nucleotide sequence encoding for amino acid residues 1 to 119 of SEQ ID NO:21.

Also intended within the scope of the invention are nucleic acid molecules
20 encoding "consensus" amino acid sequences of heavy and light chain of antibodies, including: nucleic acid molecules comprising a nucleotide sequence encoding for amino acid residues 1 to 107 of SEQ ID NO:75; nucleic acid molecules comprising a nucleotide sequence encoding for amino acid residues 1 to 107 to of SEQ ID NO:76; nucleic acid molecules comprising a nucleotide sequence
25 encoding for amino acid residues 1 to 107 of SEQ ID NO:77; nucleic acid molecules comprising a nucleotide sequence encoding for amino acid residues 1 to 119 of SEQ ID NO:78; nucleic acid molecules comprising a nucleotide sequence encoding for amino acid residues 1 to 119 of SEQ ID NO:79; nucleic acid molecules comprising a nucleotide sequence encoding for amino acid residues

1 to 119 of SEQ ID NO:80; and nucleic acid molecules comprising a nucleotide sequence encoding for amino acid residues 1 to 119 of SEQ ID NO:81.

5 Nucleic acid molecules encoding an immunologic molecule of the present invention can be used to express recombinant proteins. A nucleic acid molecule encoding an immunologic molecule of the present invention can be inserted into a vector in accordance with conventional techniques. A "vector" should be understood as a nucleic acid vehicle used for cloning or expressing a desired sequence in a host.

10 In one embodiment, the recombinant vector is capable of expressing the immunologic molecule of the present invention. A vector is said to be "capable of expressing" a polypeptide if it contains a nucleotide sequence that encodes for the polypeptide, as well as transcriptional and translational regulator information operably linked to the nucleotide sequence that encodes the polypeptide. Two
15 nucleotide sequences are said to be "operably linked" if the nature of the linkage between the two nucleotide sequences does not: result in the introduction of a frame-shift mutation; interfere with the ability of the promoter region sequence to direct the transcription of the desired sequence; or interfere with the ability of the desired sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a desired nucleotide sequence if the
20 promoter were capable of effecting transcription of that nucleotide sequence.

Once the recombinant vector is constructed, it can be introduced into a host cell, either prokaryotic or eukaryotic, by a variety of conventional techniques including transfection, transduction, electroporation, calcium-phosphate precipitation, and microinjection. Prokaryotic hosts include bacteria such as *E. coli*, *Bacillus*, *Streptomyces*, and *Salmonella*. The most preferred prokaryotic host
25 is *E. coli*. Eukaryotic hosts include yeast cells, insect cells, and mammalian cells, such as COS cells, CHO cells, and myeloma cells. In one embodiment of the invention, CHO cells are preferred.

30 In one embodiment of the invention, a nucleic acid molecule comprising a nucleotide sequence encoding for the light chain of an antibody is introduced

into a vector, and a nucleic acid molecule comprising a nucleotide sequence encoding for the heavy chain of an antibody is introducing into another vector. Both vectors are introduced into the same host cell. Alternatively, both chains could be introduced into the same vector.

5 Following expression in an appropriate host, the polypeptide can be readily isolated using standard techniques, including affinity chromatography.

 Also intended within the scope of the present invention are molecules comprising an amino acid sequence of the binding region of an immunologic molecule described herein. Molecules comprising an amino acid sequence of the
10 binding region of an immunologic molecule described herein include, but are not limited to, monoclonal antibodies, humanized antibodies, chimeric antibodies, fragments of any such antibodies, single chain antibodies, fusion proteins, and the like. Such molecules can be used in the assays and methods of treatment of the present invention described below.

15 The amino acid sequence of the binding region of the immunologic molecules of the present invention are shown in Figure 21 for the light chains and Figure 22 for the heavy chains. In Figure 21, the amino acid sequence of the binding regions of the light chains of h77A3-1 and h77A3-2 (amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:17), m77A3 (amino acid residues
20 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:9), m44C9 (amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:5), m70B11 (amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:7), the murine consensus (amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:75), the 77A3/49C9 consensus (amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID
25 NO:76) and the consensus of all light chains (amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:77) are shown in the larger boxes.

 In Figure 22, the amino acid sequence of the binding regions of the heavy chains of h77A3-1 (amino acid residues 26 to 35, 50 to 66 and 99 to 108 of SEQ ID NO:19), h77A3-2 (amino acid residues 26 to 35, 50 to 66 and 99 to 108 of
30 SEQ ID NO:21), m77A3 (amino acid residues 26 to 35, 50 to 66 and 99 to 108

of SEQ ID NO:15), m49C9 (amino acid residues 26 to 35, 50 to 66 and 99 to 108 of SEQ ID NO:11), m70B11 (amino acid residues 26 to 35, 50 to 66 and 99 to 108 of SEQ ID NO:13), the humanized consensus (amino acid residues 26 to 35, 50 to 66 and 99 to 108 of SEQ ID NO:78), the murine consensus (amino acid residues 26 to 35, 50 to 66 and 99 to 108 of SEQ ID NO:79), the 77A3/49C9 consensus (amino acid residues 26 to 35, 50 to 66 and 99 to 108 of SEQ ID NO:80), and the consensus of all the heavy chains (amino acid residues 26 to 35, 50 to 66 and 99 to 108 of SEQ ID NO:81) are shown in the overlapping boxes.

B. Assays

Methods for immunoblotting are known in the art (*see*, for example, Reed, G.L. *et al.*, *J. Immunol.* 150:4407-4415 (1993)). In a preferred method, the α 2AP is electrophoresed on a slab minigel under reducing and non-reducing conditions. The gel is electroblotted to polyvinylidene difluoride membrane. The blot is exposed to different hybridoma supernatants in the channels of a miniblottor apparatus. After washing, the bound antibody is detected by incubation with ^{125}I -goat antimouse antibody. After additional washing, the membrane is exposed in a phosphorimager (Molecular Devices, Sunnyvale, CA).

Methods for radioimmunoassays are also known. For example, the wells of a microtiter plate are coated with goat antimouse antibody. The wells are washed and blocked with BSA. The hybridoma supernatants are added to the empty wells. After incubation, the wells are washed and ^{125}I - α 2AP is added. After washing, the wells are cut and the bound antibody is measured by gamma scintillation counting. For competition assays, the wells of a microtiter plate are coated with a competing MAb. In a preferred embodiment, the binding of MAbs to ^{125}I - α 2AP (preferably, the fibrin binding region fragment of α 2AP, obtained by binding to RWR) is assayed by reverse solid-phase radioimmunoassay.

Methods for clot assays are also known (*see*, for example, Reed, G.L. III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990)). In a preferred

embodiment, plasma is mixed with ^{125}I -fibrinogen, then clotted by mixing with CaCl_2 and thrombin. Clots are compressed and washed with Tris-buffered saline to remove unbound proteins. The supernatant is removed and the clots counted in a gamma counter. To each set of duplicate clots is added, various amounts of plasminogen activator, anti-coagulant, and Tris-buffered saline containing the MAb or no MAb. The clots are incubated and at various intervals, a portion of the solution is temporarily removed and gamma-counted to determine the amount of lysis. The percent lysis may be defined at 100X (total supernatant cpm/total clot cpm).

Fibrinogen assays are known. Blood samples and platelet-poor plasma are assayed for fibrinogen by, for example, the sodium sulfite method (Rampling, M.W. and Gaffney, P.J., *Clin. Chim. Acta.* 67:43-52 (1976)).

Alpha-2-antiplasmin levels in plasma are assayed, for example, with a chromogenic substrate assay for plasmin inhibition (Stachrom kit) as described in Reed, G.L., III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990).

Statistical tests may be analyzed by, for example, a one way analysis of variance followed by a Bonferroni-Dunn procedure for multiple comparison testing.

In vivo pulmonary embolism experiments are described in Example 2, below.

C. *Methods of Treatment*

By "patient" is intended, human or nonhuman. Nonhumans include, for example, baboon, green monkey, dog, cynomolgus, marmoset, ferret, guinea pig, and gerbil.

By "clot" is intended, an *in vitro* blood or fibrin clot, or "thrombi" in a patient. Diseases treated according to the methods of his invention include, but are not limited to pulmonary thromboembolism; acute coronary syndrome, including unstable angina pectoris and non-Q-wave myocardial infarction; various

forms of thrombosis, including venous thrombosis (e.g., deep venous thrombosis), and arterial thrombosis (e.g., renal, mesenteric, and limb thrombosis); and cerebral and thrombosis embolism; renal vein and peripheral arterial thrombosis, myocardial infarction, stroke, and other thromboses. This method may also be used to treat thrombotic conditions secondary or concomitant to surgical procedures, including percutaneous transluminal coronary angioplasty, peripheral arterial angioplasty, bypass graft, and stent. The "treating" or "treatment" is by, for example, inhibiting the formation of a thrombus, dissolving a thrombus, or by enhancing fibrinolysis.

By the term "co-administration" is intended that each of the hapten-binding molecule and thrombolytic agent will be administered during a time frame wherein the respective periods of pharmacological activity overlap. The two agents may be administered simultaneously or sequentially.

The α 2AP-binding molecules of the present invention may be monoclonal antibodies or fragments thereof. It is preferable to employ the F(ab')₂ fragment of such an antibody for this purpose, in order to minimize any immunological reaction caused by the Fc portion of the immunoglobulin. Also preferred are single-chain antibodies, such as sFv. Procedures for preparing monoclonal antibodies are disclosed by Kaprowski, H. *et al.*, United States Patent No. 4,172,124, and Kohler *et al.*, *Nature* 256:495-497 (1975). The preparation of monoclonal antibodies capable of preventing the inhibition of plasmin are taught by Mimuro, J. *et al.*, *Blood* 69:446-453 (1987), and described in the examples section of the present application.

As used herein, an "antigen" is a molecule capable of being bound by an antibody such as, for example, α 2AP. In order to be used in accordance with the present invention, the "antigen-binding molecule" must be capable of binding to a plasmin inhibitor and thereby prevent such an inhibitor from forming inhibitor-plasmin complexes. Any such antigen-binding molecule may be employed in accordance with the present invention. A preferred embodiment is α 2AP-binding molecule which is capable of binding to α 2AP or fragment thereof. An especially

preferred α 2AP-binding molecule for this purpose is a monoclonal antibody. Preferred embodiments of the monoclonal antibody is 77A3, 70B11 or 49C9, described more fully below. The hybridoma producing MAb 77A3 has been deposited under the terms of the Budapest Treaty with the International
5 Depository Authority American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A., on September 20, 1996; the ATCC Accession No. is HB-12192.

Also preferred are chimeric and humanized antibodies. An especially preferred chimeric antibody for this purpose is c77A3, described more fully below.
10 Especially preferred humanized antibodies for this purpose are h77A3-1 and h77A3-2, described more fully below. Also preferred are antibody fragments and single-chain antibodies, including sFv77A3-1 and sFv77A3-2, described below.

The terms "thrombolytic agent" are meant to refer to any agent capable of either dissolving a fibrin and/or platelet clot (or thrombus), or inhibiting the
15 formation of such a clot. Examples of thrombolytic agents include fibrinolytic molecules, such as plasmin, plasminogen activator (for example, staphylokinase, streptokinase, prourokinase, urokinase, tissue-type plasminogen activator, and vampire bat plasminogen activator); anti-coagulants (for example, inhibitors of fibrin formation, such as heparin, hirudin and activated protein C; and anti-platelet
20 agents, such as ticlopidine, aspirin, and clopidigrel and inhibitors of glycoprotein IIb/IIIa function). Use of t-PA for these purposes is especially preferred. Although natural t-PA may be employed, it is preferable to employ recombinant t-PA (rt-PA). The invention may additionally employ hybrids, physiologically active fragments or mutant forms of the above thrombolytic agents. For example,
25 the term "tissue-type plasminogen activator" as used herein is intended to include such hybrids, fragments and mutants, as well as both naturally derived and recombinantly derived tissue-type plasminogen activator.

As stated, the methods of the invention comprise the administration of the α 2AP-binding molecule alone or in combination with a thrombolytic agent. When
30 administered alone the molecule enhances endogenous fibrinolysis or thrombolysis

by significantly augmenting clot lysis by endogenous plasminogen activators. Further, administration of the α 2AP-binding molecule does not increase fibrinogen consumption over that obtained with equivalent doses of t-PA alone. Thus, the present method of clot-specific inhibition of α 2AP enhances the potency of the plasminogen activator and preserves its fibrin selectivity.

Alternatively, the α 2AP-binding molecule is administered with a thrombolytic agent. In this embodiment, the α 2AP-binding molecule and the thrombolytic agent of the present invention are intended to be co-administered to the recipient. It is preferable to provide the α 2AP-binding molecule to the patient prior to the administration of the thrombolytic agent.

The α 2AP-binding molecule of the present invention is provided for the purpose of preventing the inhibition of plasmin by a plasmin inhibitor. It has been discovered that coadministration of the α 2AP-binding molecule together with a thrombolytic agent causes a synergistic effect, and thereby enhances clot lysis (thrombolysis) to a greater extent than would be expected if the effects of α 2AP-binding molecule administration and thrombolytic agent administration was merely additive.

The α 2AP-binding molecule of the present invention encompasses clot-specific inhibitors of α 2AP. It is demonstrated that the combination of t-PA and the specific inhibitors, particularly monoclonal antibodies to α 2AP, does not increase fibrinogen consumption over that obtained with equipotent doses of plasminogen activator alone. Further, clot-specific inhibition of α 2AP enhances the potency of plasminogen activators and preserves fibrin selectivity. For agents such as urokinase, which is not selective for fibrin, inhibition of clot bound α 2AP would cause a similar, or more pronounced, enhancement in potency and lead to less fibrinogen consumption as well.

Thus, the inhibition of clot-bound α 2AP enhances clot lysis by endogenous plasminogen activators. Further, when administered in combination with a thrombolytic agent, thrombolysis is significantly increased compared with the lysis achieved by equivalent doses of the thrombolytic agent alone. This increased lysis

by the combination of the thrombolytic agent and α 2AP inhibitor occurs without decreasing circulating fibrinogen or α 2AP levels. The net result is a synergistic interaction between the two agents.

When used alone, an amount of α 2AP-binding molecule capable of preventing inhibition of plasmin and thereby enhancing clot lysis when provided to a patient is a "therapeutically effective" amount. In order to enhance clot lysis and prevent clot reformation, it is desirable to provide between 3 to 300 nmole of α 2AP-binding molecule per kilogram of patient weight. This dosage may be administered, in one embodiment, over a period of between 60 to 480 minutes, by continual intravenous infusion at a rate of 0.006 to 5 nmole/kg/min. Alternatively, it is possible to provide the α 2AP-binding molecule in an intravenously injectable bolus at a dose of between 3 to 600 nmole/kg, and most preferably between 30 to 60 nmole (of α 2AP-binding molecule) per kilogram of patient weight. If the α 2AP-binding molecule is provided in this manner, a single bolus is sufficient to prevent potential clot reformation. The α 2AP-binding molecule of the present invention may be dissolved in any physiologically tolerated liquid in order to prepare an injectable bolus. It is preferable to prepare such a bolus by dissolving the α 2AP-binding molecule in normal saline.

When the α 2AP-binding molecule capable of preventing inhibition of plasmin is co-administered with a thrombolytic agent, it is desirable to provide 3 to 300 nmole of α 2AP-binding molecule per kilogram of patient weight. This dosage may be administered, in one embodiment, over a period of 60 to 480 minutes, by continuous intravenous infusion. Alternatively, it is possible to provide the α 2AP-binding molecule in an intravenously injectable bolus at a dose of between 3 to 600 nmole/kg, and most preferably between 30 to 60 nmole/kg of patient weight. An amount of thrombolytic agent capable of causing such lysis is a "therapeutically effective" amount. It is desirable to provide between 0.01 to 3.0 mg per kilogram of patient weight. In one embodiment, the thrombolytic agent is provided over a prolonged period (i.e., from about 180 to about 1440 minutes). In a preferred embodiment, the thrombolytic agent of the present

invention is provided as an intravenously injected bolus containing between 0.5 to 1.0 mg/kg, and most preferably between 0.5 to 0.75 mg/kg. For example, for pulmonary embolism, the dosage of t-PA by continuous infusion is ~100 mg for 2 hours (Goldhaber, S.C. *et al.*, *Lancet* 341:507 (1993)). The dosage to be used of thrombolytic agent of the present invention is generally known in the art (*see, e.g., Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 3rd ed. Philadelphia, PA (1994)).

The thrombolytic agent of the present invention may be dissolved in any physiologically tolerated liquid in order to prepare an injectable bolus. It is, however, preferable to prepare such a bolus by dissolving the thrombolytic agent in normal saline.

A patient treated according to the preferred embodiment will, therefore, receive an intravenously injected bolus of the α 2AP-binding molecule in combination with an intravenously injected bolus of the thrombolytic agent. This preferred treatment minimizes the amount of t-PA required for thrombolysis, thus reducing the extent of fibrinogen breakdown and lessening any tendency for general hemorrhage. Importantly, the use of the preferred treatment results in the dissolution of the occluding thrombus at a rate which greatly exceeds the rate of thrombus dissolution when either the α 2AP-binding molecule or the thrombolytic agent is provided by infusion alone. Additionally, the risk of reocclusion is substantially reduced.

In previous models of fibrinolysis (3), the chief role assigned to α 2AP has been to inactivate circulating plasmin and prevent a systemic lytic state. Thus, it may be surprising that an α 2AP inhibitor can increase clot lysis without increasing fibrinogenolysis. This marked amplification of thrombolysis by α 2AP inhibitor underscores the importance of fibrin bound α 2AP in regulating fibrinolysis. Since the subject antibodies augment clot lysis by a fibrin-selective agent such as t-PA as well as that by the nonselective activators urokinase and streptokinase, it appears that fibrin-bound α 2AP plays a critical role in determining the rate of lysis by any exogenous plasminogen activator.

These unexpected findings are important because it had previously not been possible to accelerate the rate of clot lysis without increasing the tendency to hemorrhage. The preferred embodiment, therefore, provides a method of treatment in which the administration of a bolus of a α 2AP-binding molecule in combination with the administration of a bolus of a thrombolytic agent are capable of dissolving an occluding thrombus at a faster rate than can be obtained when either compound is administered alone. Moreover, the preferred embodiment accomplishes this goal while minimizing both fibrinogen breakdown and the risk of reocclusion. Thus, the combination of agents can significantly increase the potency and specificity of thrombolytic therapy.

As would be apparent to one of ordinary skill in the art, the required dosage of the anti- α 2AP binding molecule or thrombolytic agent will depend upon the severity of the condition of the patient, and upon such criteria as the patient's height, weight, sex, age, and medical history.

The α 2AP-binding molecule or thrombolytic agent of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, such as by admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described, for example, in *Remington's Pharmaceutical Sciences*, 16th Ed., Osol, A., ed., Mack, Easton PA (1980). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the α 2AP-binding molecule or thrombolytic agent, either alone, or with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved by the use of polymers to complex or absorb the α 2AP-binding molecule or thrombolytic agents of the present invention. The controlled delivery may be exercised by selecting appropriate macromolecules (for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate). The rate of drug release may also be controlled by altering the

concentration of such macromolecules. Another possible method for controlling the duration of action comprises incorporating the therapeutic agents into particles of a polymeric substance such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, it is possible to entrap the therapeutic agents in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethylcellulose or gelatin-microcapsules or poly(methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, nanocapsules, or in macroemulsions. Such teachings are disclosed in *Remington's Pharmaceutical Sciences*, 16th Ed., Osol, A., ed., Mack, Easton PA (1980).

The thrombolytic agent or α 2AP-binding molecule may be provided to a patient by means well known in the art. Such means of introduction include oral means, intranasal means, subcutaneous means, intramuscular means, intravenous means, intra-arterial means, or parenteral means. In one preferred method of treatment for pulmonary embolism, myocardial infarction, thrombosis or stroke, a patient is provided with a bolus (intravenously injected) containing between 0.5 to 1.0 mg/kg of a thrombolytic agent.

Generally, the results reported herein demonstrate that an inhibitor, particularly a monoclonal antibody, can be used to augment the catalytic function of an enzyme by neutralizing an inhibitor of that enzyme. This approach can be applied to biological processes which are tightly governed by inhibitors. Because coagulation is a finely balanced system in which the effects of enzymes (generally serine proteases) are pitted against the effects of inhibitors, frequently serpins (serine protease inhibitors) pathological alterations in clotting can be treated by augmenting enzyme activity or by neutralizing an inhibitor.

Having now generally described this invention, the same will be better understood by reference to certain specific examples which are included herein for purposes of illustration and are not intended as limiting.

Example 1

Preparation of an Antibody Directed to Alpha-2-antiplasmin

A. Monoclonal Antibody Production, Purification and Characterization

5 Two Balb/C mice were immunized subcutaneously with 25 μ g of purified human α 2AP fragments derived from the trypsin digest of a human plasma clot. The α 2AP fragments were affinity purified with a SEPHAROSE-coupled monoclonal antibody, RWR (Reed, G.L. III *et al.*, *Trans. Assoc. Am. Phys.* 101:250-256 (1988); U.S. Patent No. 5,372,812, issued December 13, 1994),
10 against human α 2AP. Mice were initially immunized with complete Freund's adjuvant and boosted 90 days later with 50 μ g of α 2AP fragment in incomplete Freund's adjuvant. The antisera titer was tested in a solid-phase radioimmunoassay (Reed, G.L. III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990)) with α 2AP immobilized in the wells of a microtiter plate. Four days
15 before fusion, the mouse with the highest titer of α 2AP antibody was hyperimmunized with 100 μ g α 2AP intraperitoneally. Somatic cell fusion was performed as described (Galfre, G. and Milstein, C., *Meth. Enzymol.* 73:3-46 (1981)).

Hybridomas were tested for the production of antibodies to the α 2AP
20 fragment and for their ability to inhibit α 2AP as described in Reed, G.L. III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990). The binding of monoclonal antibodies (MAbs) to 125 I- α 2AP was tested in a solid-phase radioimmunoassay. Wells of a microtiter plate were coated with goat antimouse antibody (25 μ l, 5 μ g/ml) for 2 hours. The wells were rinsed and nonspecific protein binding sites
25 were blocked with 1% bovine serum albumin in Tris-buffered saline, pH 7.4, for 1 hour. After a wash, 25 μ l of hybridoma supernatant was added to the wells and incubated for 1 hour. The wells were rinsed and 125 I- α 2AP was added (25 μ l,

60,000 cpm) for 1 hour. The ^{125}I - $\alpha 2\text{AP}$ was then removed and the wells were rinsed and gamma-counted.

Cloned hybridomas (limiting dilution) were expanded into ascites in pristane-primed Balb/C mice. Antibodies were purified from filtered ascites by precipitation with 40% ammonium sulfate, dialysis into 10 mM KH_2PO_4 , pH 7.2, and ion-exchange chromatography on DEAE-AFFIGEL BLUE SEPHAROSE (BioRad, Hercules, CA) with a linear gradient from 0 to 100 mM NaCl.

B. Immunoblotting

These were performed largely as described in Reed, G.L. *et al.*, *J. Immunol.* 150:4407-4415 (1993). Purified human $\alpha 2\text{AP}$ (5 μg , American Diagnostica, Greenwich, CT) was electrophoresed in a large single sample lane on a 12% slab minigel (BioRad, Hercules, CA) under reducing and non-reducing conditions. The sample was electroblotted (Kyhse-Anderson, 1984) to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and nonspecific protein binding sites were blocked with 5% dry milk. The blots were exposed to different hybridomas supernatants for 1 hour in the channels of a miniblotted apparatus (Immunetics, Cambridge, MA). After washing, the bound antibody was detected by incubation with ^{125}I -goat antimouse antibody (1.5 million cpm/membrane). After additional washing, the membranes were exposed in a phosphorimager (Molecular Devices, Sunnyvale, CA).

C. Radioimmunoassays

Wells of a microtiter plate were coated with goat antimouse antibody (25 μl , 5 $\mu\text{g}/\text{ml}$) for 2 hours at 21°C. They were washed and blocked with 1% BSA (bovine albumin serum) for 1 hour. To the empty wells in duplicate were added 25 μl of hybridoma supernatants. After incubation for 1 hour the wells were washed and 25 μl of ^{125}I - $\alpha 2\text{AP}$ was added to the wells for another hour.

After washing the wells were cut and the bound antibody measured by gamma scintillation counting.

Competition radioimmunoassays were performed by coating wells of a microtiter plate with 25 μ l of purified MAb (70B11) in duplicate (10 μ g/ml) for 1 hour. The wells were washed and blocked with 1% BSA for 1 hour. After washing, 25 μ l of a competitor MAb, same MAb or negative control MAb was added to different wells (50 μ g/ml) followed by 25 μ l of 125 I- α 2-antiplasmin (100,000 cpm). After 1 hour incubation, the wells were washed, cut and the radioactivity was measured in a gamma scintillation counter.

D. Plasma Clot Lysis Assays

These were performed largely as described in Reed, G.L. III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990). Pooled fresh frozen plasma was obtained from 5 random donors to the Massachusetts General Hospital Blood Bank. The plasma was mixed with 125 I-fibrinogen to achieve \sim 1,000 cpm/ μ l. The plasma was clotted for 1 hour at 37°C in a 12 x 65 mm test tube by mixing 50 μ l with 50 μ l of CaCl_2 (5 mM final) and thrombin (1 U/ml). Clots were compressed and washed in 1 ml Tris-buffered saline (pH 7.4) to remove unbound proteins. The supernatant was removed and the clots were counted in a gamma counter. To each set of duplicate clots was added 100 μ l containing various amounts of urokinase, 100 μ l of pooled plasma containing 1 u/ml of hirudin and 100 μ l of Tris-buffered saline containing 7 μ g (Figure 4) or 21 μ g (Figure 5) of MAb or no MAb. The clots were placed in a 37°C water bath and at various intervals 100 μ l of solution was temporarily removed and gamma-counted to determine the

amount of lysis. The percent lysis was defined at $100 \times (\text{total supernatant cpm} \div \text{total clot cpm})$.

E. Results

Three hybridomas were selected that appeared to inhibit $\alpha 2\text{AP}$ function in screening assays. The serotypes of these MAbs were: 49C9 (Ig γ 2aK), 70B11 (Ig γ 1K), and 77A3 (Ig γ 2aK). Figure 1 compares the binding of these MAbs to ^{125}I - $\alpha 2\text{AP}$ in a reverse solid-phase assay. When compared to the original $\alpha 2\text{AP}$ inhibitor RWR, these MAbs bound with greater avidity. To determine if the MAbs bound to the same epitopes, competition assays is shown for 70B11 in Figure 2. Compared to the negative control, anti-digoxin MAb, RWR had no significant inhibitory effects on the binding of ^{125}I - $\alpha 2\text{AP}$ to immobilized 70B11. In contrast, when 70B11 was used as a competitor, it completely inhibited the binding of ^{125}I - $\alpha 2\text{AP}$ to immobilized 70B11, as expected. However, 49C9 and 77A3 were also excellent competitors as well. The results of these assays are shown in tabular form in Table 1, below. MAbs 49C9, 70B11, 77A3 also fully inhibited the binding of each other to ^{125}I - $\alpha 2\text{AP}$, but they had no inhibitory effects on the binding of RWR. The converse was also true, RWR as a competitor had no effect on the binding of ^{125}I - $\alpha 2\text{AP}$ to the other MAbs. This indicated that MAbs 49C9, 70B11 and 77A3 competed for binding to the same epitope, while RWR appeared to bind to a separate region of $\alpha 2\text{AP}$.

To determine if the MAbs recognized a continuous or discontinuous epitope in $\alpha 2\text{AP}$, immunoblotting experiments were performed with denatured and reduced $\alpha 2\text{AP}$. In these studies RWR bound well to denatured and reduced $\alpha 2\text{AP}$, indicating that it recognized an epitope which was not affected by boiling with SDS, nor reduction of disulfide bonds. In contrast, MAbs 49C9, 70B11 and 77A3 did not bind to denatured $\alpha 2\text{AP}$, suggesting that they recognize a conformation-dependent epitope.

Clot lysis assays were performed to examine the relative potency of these MAbs in amplifying the fibrinolysis by urokinase. Figure 3 compares the amount of lysis achieved by 7 μ g of different purified MAbs (or TBS alone) as a function of dose of urokinase. Compared to urokinase alone (TBS) or urokinase with the control antidigoxin MAb, RWR, 49C9, 70B11 and 77A3 all accelerate clot lysis. However, 49C9, 70B11 and 77A3 appear to be significantly more potent than RWR in these assays. To examine the increase in fibrinolytic potency of urokinase achieved by one of these antibodies, dose response studies were performed in the absence or presence of MAb 77A3. Figure 4 shows that MAb 77A3 markedly increases the potency of lysis of urokinase by roughly 100-fold.

As a means of further discriminating among the functional and epitope binding specificities of these MAbs, their ability to inhibit the α 2AP from different animal species in plasma clot lysis assays was examined. The results of these assays are summarized in Table 2, below. In the different species of animal plasmas tested, RWR appeared to inhibit only human α 2AP. In contrast, the other MAbs showed a broader species cross-reactivity and ability to inhibit nearly all primate and some non-primate α 2APs.

Table 1 Effect of Different MAb Inhibitors on the binding of ^{125}I - $\alpha 2\text{AP}$ to Immobilized MABs.

	Immobilized MAb			
Inhibitor	RWR	49C9	70B11	77A3
RWR	+	-	-	-
49C9	-	+	+	+
70B11	-	+	+	+
77A3	-	+	+	+
anti-digoxin	-	-	-	-
To wells of a microtiter plate containing an immobilized MABs was added ^{125}I - $\alpha 2\text{AP}$ and a different competitor MABs. A "+" indicates that the competitor inhibited the binding of ^{125}I - $\alpha 2\text{AP}$ to the plate, whereas a "-" indicates that there was no inhibition.				

Table 2 The cross reactivity of MABs with different $\alpha 2$ -antiplasmins.

Species	RWR	49C9	70B11	77A3
HUMAN	++	++	++	++
Baboon	-	++/+	++/+	++
Grn Monkey	-	++	++	++
Dog	-	+	+/-	+
cynomolgus	-	++	++	++
marmoset	-	+	+	+
ferret	-	+/++	+/-	+/++
guinea pig	-	-	+/-	+/-
gerbil	-	-	-	-
The crossreactivity of each MAB was determined by its ability to accelerate the lysis of that species' plasma clots. A "-" indicated that the MAB did not accelerate plasma clot lysis, a "+" indicated modest effects, and "++" indicates significant acceleration of plasma clot lysis (i.e., significant functional crossreactivity).				

Example 2

In Vivo Study of Pulmonary Embolism

A. Materials

Materials were obtained from the following suppliers: rt-PA with a specific activity of 580,000 IU/mg, Genentech (South San Francisco, California); ketamine (100 mg/ml), Fort Dodge Laboratories (Fort Dodge, Iowa); acepromazine maleate, Fermenta Animal Health Co. (Kansas City, MO); heparin (1000 U/ml), Elkins-Sinn Inc. (Cherry Hill, NJ); sodium iodide, Aldrich Chemical Co. (Milwaukee, WI); calcium chloride, Mallinckrodt (Paris, Kentucky); normal saline for intravenous use, Travenol Laboratories (Deerfield, IL); α 2AP assay kit, Stachrom (Asnières, France); purified α 2AP and fibrinogen, American Diagnostica (Greenwich, CT); goat antimouse antibody, Cappel Organon Technika (Durham, NC); human plasma pooled from random donors, Massachusetts General Hospital (Boston); bovine thrombin, Parke-Davis (Morris Plains, NJ); Na^{125}I , Dupont-NEN (Cambridge, MA); Bard Parker surgical blade, Becton Dickinson (Franklin Lake, NJ); 4.0 silk sutures, American Cyanamid Co. (Danbury, CT); SURFLO IV catheter and 20 gauge 1-1/4-inch VENOJECT tubes with K_3EDTA , Terumo Medical Corp. (Elkton, MD); sterile three-way stopcock, Mallinckrodt Critical Care (Glens Falls, NY); auto syringe infusion pump, Baxter Health Care Corp. (Hooksett, NH); infusion pump tubing and microbore 60-inch extension set, McGaw of Puerto Rico (Sabana Grand, Puerto Rico); surgical instruments, VWR (Boston); tubing, Namic (Glens Falls, NY); ferrets (~.8– 1 kg), Marshall Farms (New York, NY); aprotinin, Sigma (St. Louis, MO); and microcentrifuge tubes, National Scientific Supply Co. (San Rafael, CA).

B. *In Vitro Clot Lysis Assays*

Pooled, fresh-frozen, citrated ferret plasma (1100 μ l) was mixed with 15 μ l of 125 I-labeled human fibrinogen (~40,000 cpm/clot). Ferret plasma (35 μ l) was mixed with 35 μ l of Tris-buffered saline (TBS) containing 10 mM CaCl_2 and thrombin (1 U/ml) in twelve 65-mm plastic tubes and clotted for 1 hour at 37°C. The clots were washed in TBS, the supernatant was removed, and then 100 μ l of TBS or 25 μ g of purified MAb (RWR or 77A3) was added to tubes in duplicate. Clot lysis was initiated by adding 0.1 U of rt-PA per tube. The clots were incubated at 37°C for 5 hours and the amount of lysis was determined by sampling for the release of radiolabeled fibrin degradation products into the supernatant, as described (Reed, G.L. III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990)).

C. *Pulmonary Embolism Experiments*

Male ferrets were anesthetized by intramuscular injection (0.4 ml) of a mixture of ketamine and acepromazine (two parts acepromazine [10 mg/ml] to one part ketamine [100 mg/ml]). Intraperitoneal injections were repeated as necessary to keep the animals anesthetized. After an anterior midline incision had been made in the neck, the jugular vein and the carotid artery were exposed by blunt dissection and cannulated with 20G catheters that were secured at the proximal and distal ends with 4-0 silk sutures. The catheters were capped with three-way stopcocks.

Pooled, citrated human plasma was mixed with 125 I-fibrinogen to achieve ~1,000,000 cpm/ml. Individual clots were formed by mixing 125 I-fibrinogen-labeled plasma (45 μ l) with 2.5 μ l of bovine thrombin (100 U/ml) and 2.5 μ l of calcium chloride (0.4 M). These clots were incubated at 37°C for 90 minutes, compressed, and washed thoroughly with saline three times to remove unbound proteins. The radioactive content of the clots was measured in a gamma counter

immediately before clot injection. Blood samples were drawn at base line and at the end of the experiment. Sodium iodide (10 mg) was injected to block thyroid uptake. Clots were embolized into the lungs by injection through the internal jugular vein. Ferrets weighing less than 1 kg received three clots; those weighing 1 kg or more received four clots. Successful embolization was evidenced by the accumulation of radioactivity in the thorax. After the clots had been injected, the ferrets were turned on their sides to ease breathing.

All animals received weight-adjusted heparin at 100 U/kg (bolus), a dose sufficient to keep the activated partial thromboplastin time (aPTT) above 150 seconds throughout the procedure. The α 2AP inhibitor (sterile-filtered, 14 mg/ml in saline) or a purified control MAb (antidigoxin) was given intravenously as a single dose (22.5 mg/kg). The rt-PA was given as a continuous infusion over 2 hours (1 or 2 mg/kg in 5 ml normal saline). Animals were observed for a total of four hours after pulmonary embolization and then killed by lethal injection of anesthesia or by CO₂ inhalation. The thorax was dissected and all intrathoracic structures were removed for gamma counting to detect residual thrombi. The percentage of clot lysis was determined for each ferret by dividing the total residual radioactivity in the *thorax* (cpm) by that in the initial thrombi.

This experimental protocol was approved by the Harvard Medical Area Standing Committee on Animals. The Harvard Medical School animal management program is accredited by the American Association of Laboratory Animal Care, and the procedures were conducted in accordance with National Institutes of Health standards, as set forth in the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. [NIH] 85-23, revised 1985), the Public Health Service Policy on the Humane Care and Use of Laboratory Animals by Awardee Institutions, and the NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

D. Statistical Tests

The data were analyzed by a one way analysis of variance followed by a Bonferroni-Dunn procedure for multiple comparison testing.

E. Fibrinogen Assays

5 Blood samples were collected on K₃EDTA (0.15% solution final) with aprotinin (50 kallikrein U/ml). Platelet-poor plasma was obtained by centrifugation of whole blood (Mustard, J.F. *et al.*, *Meth. Enzymol.* 169:3-11 (1989)) and assayed for fibrinogen by the sodium sulfite method (Rampling, M.W. and Gaffney, P.J., *Clin. Chim. Acta.* 67:43-52 (1976)).

10 **F. α 2-Antiplasmin Assays**

To measure α 2AP levels, we collected ferret blood on sodium citrate (1/10 volume) and centrifuged it to obtain plasma (Mustard, J.F. *et al.*, *Meth. Enzymol.* 169:3-11 (1989)). The plasma was tested for functional α 2AP with a chromogenic substrate assay for plasmin inhibition (Stachrom kit) as described
15 (Reed, G.L. III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990)).

G. Results

From a panel of hybridomas we selected 77A3, a MAbs that bound tightly to human α 2AP. MAbs 77A3 was purified from mouse ascites by ion exchange chromatography, and its purity was confirmed by SDS-polyacrylamide gel analysis
20 (Figure 5). To study the role of α 2AP in experimental pulmonary embolism *in vivo*, we tested purified 77A3 in several different animal plasma clot lysis assays to determine whether it could bind and inhibit a non-human α 2AP. Of various small animal plasmas tested (e.g. hamster, gerbil, guinea pig, rat, etc.), 77A3

significantly crossreacted with ferret plasma. Figure 6 compares the lytic effects of 77A3 with those of another MAb inhibitor of human α 2AP, RWR (Reed, G.L. III *et al.*, *Trans. Assoc. Am. Phys.* 101:250-256 (1988); U.S. Patent No. 5,372,812, issued December 13, 1994), and with buffer alone. Figure 6 shows that in comparison with the control (buffer alone), 77A3 accelerated the lysis of ferret plasma clots induced by a low dose of rt-PA (0.1 unit). In contrast, RWR, which inhibits human α 2AP (Reed, G.L. III *et al.*, *Trans. Assoc. Am. Phys.* 101:250-256 (1988); U.S. Patent No. 5,372,812, issued December 13, 1994) but does not crossreact with nonhuman α 2AP, had no detectable effect. This experiment indicated that 77A3 inhibited ferret α 2AP and amplified ferret clot lysis *in vitro*.

The cross-reactivity of 77A3 allowed us to investigate the role of α 2AP in a ferret model of pulmonary embolism. In humans, pulmonary embolism is usually treated with heparin (Goldhaber, S., *Chest* 107:45S-51S (1995)). Consequently, ferrets were treated with a weight-adjusted bolus dose of heparin at 100 U/kg. This dose was sufficient to keep the aPTT above 150 seconds throughout the experiment (n=3). To investigate the effects of intravenous MAb 77A3 on the activity of α 2AP in the blood, we selected a dose, 22.5 mg/kg, that was in molar excess to the level of ferret α 2AP. Our *ex vivo* measurements of ferret α 2AP activity, 1 and 4 hours after intravenous dosing, showed that ~75% of ferret α 2AP activity was inhibited at this dose (Figure 7, n=2).

Using heparin at 100 U/kg and 77A3 at 22.5 mg/kg, we then investigated the effect of these agents and rt-PA on the lysis of pulmonary emboli (Figure 8). All animals received heparin. Control animals (n=8), which received no rt-PA, showed 15.6 \pm 10.5% (mean \pm SD) lysis of their pulmonary emboli. Animals receiving rt-PA at 1 mg/kg (n=4) over 2 hours showed 38.5 \pm 6.3% lysis, which was significantly greater than lysis obtained in those receiving heparin alone ($P<.01$). Similarly, animals receiving rt-PA at 1 mg/kg and a control (antidigoxin) MAb (n=3) showed 35.2 \pm 4.6% lysis. Ferrets treated with rt-PA at 2 mg/kg (n=4) showed a minimal increase in lysis over those treated at 1 mg/kg (45.0 \pm 6.5% vs

38.5±6.3%, $P<.05$). However, animals receiving rt-PA at 1 mg/kg together with the α 2AP inhibitor (n=4) showed greater lysis (56.2±4.7%) than those receiving an equivalent dose of rt-PA alone ($P<.01$), with or without the control (antidigoxin) MAb ($P<.01$), or those receiving twice the dose of rt-PA alone ($P<.05$).

In addition to inhibiting plasmin on the thrombus surface, α 2AP and other inhibitors inactivate plasmin in the blood (Collen, D., *Eur. J. Biochem.* 69:209-216 (1976); Moroi, M. and Aoki, N., *J. Biol. Chem.* 251:5956-5965 (1976); Mullertz, S. and Clemmensen, I., *Biochem J.* 159:545-553 (1976)). We measured fibrinogen levels in the blood to determine if inhibition of α 2AP led to nonspecific plasminolysis of a circulating clotting factor. Figure 9 shows residual fibrinogen levels expressed as a function of their initial values in four treatment groups. In animals that received no rt-PA, fibrinogen levels varied moderately but did not diminish during the experiment. Ferrets receiving 1 mg/kg and 2 mg/kg of rt-PA alone showed no significant change in fibrinogen level. Similarly, animals receiving the combination of rt-PA and the α 2AP inhibitor showed no detectable change in circulating fibrinogen levels.

H. Discussion

Clinical and experimental studies suggest that pulmonary emboli and venous thrombi resist endogenous fibrinolysis and lysis induced by plasminogen activators (Goldhaber, S., *Chest* 107:45S-51S (1995); Goldhaber, S.Z. *et al.*, *Lancet* 2:886-889 (1986); The Urokinase Pulmonary Embolism Trial, *Circulation* 47:1-108 (1973); Goldhaber, S.Z. *et al.*, *Am. J. Med.* 88:235-240 (1990); Goldhaber, S.Z. *et al.*, *Lancet* 341:507-511 (1993)). This resistance to lysis is due in part to specific molecular factors in the thrombus that act to oppose fibrinolysis. During thrombus formation, α 2AP is covalently crosslinked to fibrin by activated factor XIII (Sakata, Y. and Aoki, N., *J. Clin. Invest.* 69:536-542 (1982)). Studies *in vitro* indicate that when α 2AP in the clot is absent or inhibited by MAbs, clots

undergo spontaneous lysis (Aoki, N. *et al.*, *Blood* 62:1118-1122 (1983); Miles, L.A. *et al.*, *Blood* 59:1246-1251 (1982); Reed, G.L. III *et al.*, *Trans. Assoc. Am. Phys.* 101:250-256 (1988); Reed, G.L. III *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1114-1118 (1990)). Conversely, when levels of α 2AP in clots are increased by supplementation *in vitro*, fibrinolysis is inhibited (Sakata, Y. and Aoki, N., *J. Clin. Invest.* 69:536-542 (1982)). In the present study we investigated the hypothesis that α 2AP plays a major regulatory role in fibrinolysis and that it contributes to the thrombus resistance obtained in pulmonary embolism.

We measured the effect of rt-PA, with and without α 2AP inhibition, on the net lysis of pulmonary emboli in ferrets. Because heparin is the established therapy for humans with pulmonary embolism, we considered animals treated with heparin alone as the control group. The weight-adjusted bolus dose of heparin given to the ferrets was sufficient to maintain a high level of anticoagulation throughout the experiment. In animals treated with rt-PA, at a dose comparable to that used in humans (1 mg/kg), lysis of pulmonary emboli was enhanced significantly in comparison with lysis in animals treated with heparin alone. Increasing the dose of rt-PA to 2 mg/kg, a dose higher than is safe in humans, led to a minimal increase in lysis. A similar plateau in the dose response for t-PA-induced lysis has been noted in experimental studies of pulmonary embolism in dogs (Werier, J. *et al.*, *Chest*. 100:464-469 (1991)). However, specific inhibition of α 2AP markedly potentiated the lysis of experimental pulmonary emboli by rt-PA (1 mg/kg), causing significantly more lysis than was seen in ferrets treated with the same dose of rt-PA: alone or with a control MAb, the lysis achieved with α 2AP inhibition was also greater than that achieved in ferrets treated with high-dose rt-PA (2 mg/kg). At the same time, despite the higher total lysis obtained in animals treated with the α 2AP inhibitor, there was no significant consumption of circulating fibrinogen. In these studies of experimental pulmonary embolism, α 2AP played an important role in thrombus resistance to lysis induced by rt-PA. Further studies will be necessary to establish the relative quantitative roles of circulating and thrombus bound α 2AP in this process.

Besides α 2AP, other molecular factors may regulate the thrombus resistance of pulmonary emboli. A leading candidate is PAI-1, a serine protease inhibitor of t-PA and urinary-type plasminogen activator (u-PA or urokinase) (Stringer, H.A. and Pannekoek, H., *J. Biol. Chem.* 270:11205-11208 (1995); Carmeliet, P. *et al.*, *J. Clin. Invest.* 92:2756-2760 (1993); Lang, I.M. *et al.*, *Circulation* 89:2715-2721 (1994); Marsh, J.J. *et al.*, *Circulation* 90:3091-3097 (1994)). Unlike α 2AP, PAI-1 is not specifically crosslinked to fibrin in the thrombus, although it has been shown to bind to fibrin *in vitro* (Stringer, H.A. and Pannekoek, H., *J. Biol. Chem.* 270:11205-11208 (1995)). By adding recombinant PAI-1 to developing thrombi, Marsh *et al.* (Marsh, J.J. *et al.*, *Circulation* 90:3091-3097 (1994)) have shown that PAI-1-enriched clots can suppress the spontaneous lysis of pulmonary emboli in a canine model; however, the role of PAI-1 in the lysis of autologous thrombi was not investigated. Pathologic studies of pulmonary emboli extracted by thrombectomy have suggested that PAI-1 expression increases in the endothelial cells at the margins of fresh thrombi but is not detectable in the thrombi themselves (Lang, I.M. *et al.*, *Circulation* 89:2715-2721 (1994)). Since PAI-1-deficient mice (by gene deletion) are less likely than regular mice to develop venous thrombosis induced by endotoxin (Carmeliet, P. *et al.*, *J. Clin. Invest.* 92:2756-2760 (1993)), the expression of PAI-1 in endothelial cells at the margin of the developing thrombus may be functionally important. Nonetheless, the role of PAI-1 in thrombus resistance to pharmacologic plasminogen activators is less clear: in patients given t-PA, the inhibitory capacity of PAI-1 is overwhelmed completely (Lucore, C.L. and Sobel, B.E., *Circulation* 77:660-669 (1988)), and thrombus resistance is also observed in patients given streptokinase, against which PAI-1 has no effect.

Another potential cause of thrombus resistance in pulmonary embolism is activated factor XIII. Several studies *in vitro* suggest that this coagulation enzyme renders the fibrin in clots more resistant to degradation by plasmin by crosslinking fibrin chains together and by crosslinking α 2AP to fibrin. (Sakata, Y. and Aoki, N., *J. Clin. Invest.* 69:536-542 (1982); Robbie, L.A. *et al.*, *Thromb.*

Haemostas. 70:301-306 (1993); Francis, C.W. and Marder, V.J., *J. Clin. Invest.* 80:1459-1465 (1987); Jansen, J.W.C.M. *et al.*, *Thromb. Haemostas.* 57:171-175 (1987); Reed, G.L. *et al.*, *Trans. Assoc. Am. Phys.* 104:21-28 (1991)) However, little is known about activated factor XIII and thrombus resistance *in vivo*. This is probably due to the fact that a potent inhibitor of factor XIII function has only recently become available (Reed, G.L. and Lukacova, D., *Thromb. Haemostas.* 74:680-685 (1995)). One study has suggested that when factor XIII is partially inhibited, coronary thrombi lyse at accelerated rates in response to t-PA (Shebuski, R.J. *et al.*, *Blood* 75:1455-1459 (1990)). This observation argues that factor XIII, through its effects on fibrin-fibrin and α 2AP-fibrin crosslinking, also contributes to thrombus resistance.

Improving the lysis of thrombi in patients with pulmonary embolism and deep venous thrombosis remains a challenge. Unfortunately, increasing the dose of plasminogen activators is not a promising approach. High dose t-PA has been associated with an unacceptable increase in the risk of cerebral bleeding (Passamani, E. *et al.*, *J. Am. Coll. Cardiol.* 10:51B-64B (1987)). In addition, in the present study and others (Werier, J. *et al.*, *Chest.* 100:464-469 (1991)), high-dose t-PA (≥ 2 mg/kg) produced only minimal increases in net lysis. The current FDA-approved doses of urokinase and streptokinase cause plasminogen "depletion"; thus, increasing the doses of these agents is also not likely to have an effect on net lysis (Onundarson, P.T. *et al.*, *J. Lab. Clin. Med.* 120:120-128 (1992)). Several potent inhibitors of thrombin generation and activity are under development. Although these agents may further reduce the formation of new thrombi, they will not directly improve lysis of the large thrombi that typically exist in patients at the time they are diagnosed. These considerations suggest that fundamental insights into the molecular factors that oppose physiologic or pharmacologic lysis in thrombi will be necessary to spark improved treatments for venous thromboembolism. The results of the present study indicate that α 2AP is a major contributor to thrombus resistance in experimental pulmonary embolism,

and they suggest that inhibiting α 2AP might improve lysis in patients with thrombotic disease.

Example 3

Cloning and Sequencing of Antibody cDNA

A. Amino Terminal Sequences of Antibodies

Monoclonal antibodies (49C9, 70B11 and 77A3) were expanded into ascites and purified by ion exchange chromatography on DEAE Affigel Blue or by protein A agarose as described in Lukacova, D. *et al.*, *Biochemistry* 30:10164-10170 (1991). The purified MAbs (15 μ g) were subjected to SDS-PAGE on 10% minigels (BioRad, Hercules, CA). The protein samples were electroblotted to PVDF membranes (Millipore, Bedford, MA) using semi-dry technique (Kyhse-Anderson, J., *J. Biochem. Biophys. Meth.* 10:203-209 (1984)) at 4°C for 2 hrs at 75 milliamps (Millipore electroblotter). The bands were stained with Ponceau Dye (Sigma, St. Louis) and excised. The amino terminal sequences of the light chain of the antibodies are shown in Figure 10 (SEQ ID NOS: 1-3).

B. Molecular Cloning of Antibody cDNA

Cloned hybridoma cell lines 49C9, 70B11 and 77A3 were grown in 150 mm tissue culture plates in 20% fetal bovine serum in Dulbecco's modified Eagle's medium with 4.5 g/l of glucose and penicillin and streptomycin. The cells were harvested and centrifuged at 1200 rpm for 7 min. The cell pellet was resuspended in sterile phosphate buffered saline (pH 7.4) and re-centrifuged. Then 5 ml of RNazol (Teltest, Friendswood, TX) was added and the pellet was homogenized for 2 min. Chloroform (500 μ l) was added and the mixture was vortexed and left to incubate on ice for 15 min. The samples were centrifuged at 12,000 rpm for 15

min. The aqueous layer was mixed with 4.5 ml of isopropanol and vortexed. The mixture was precipitated at -70°C for 90 min. and recentrifuged at 12,000 rpm for 15 min. The pellet was washed in 2 ml of 70% ethanol in DEPC-treated water. After repeat centrifugation, the supernatant was removed and the pellet air-dried.
5 The pellet was dissolved in 200 µl of diethyl-pyrocabonate (DEPC)-treated water and 20 µl of 3 M NaCl and 800 µl of ethanol were added. The mRNA was precipitated overnight at -70°C and the pellet resuspended in DEPC-water.

The cDNA corresponding to the light and heavy chain sequences were isolated by primer guided reverse transcription followed by polymerase chain
10 reaction as described (Gene Amp Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin-Elmer Cetus, San Francisco, CA). The light chain mRNA was primed for reverse transcription with a 3' primer (5' N6GAATTCACTGGATGG TGGGAAGATGGA 3' (SEQ ID NO:22)) corresponding to the constant region of the light chain (Coloma, M.J., *et al.*, *Biotechniques* 11:152-154, 156 (1991))
15 and the heavy chain was primed with a 3' primer (5' N6GAATTCA(TC) CTCCACACACAGG(AG)(AG)CCAGTGGATAGAC 3' (SEQ ID NO:23)) corresponding to the constant region of the heavy chain (Coloma, M.J., *et al.*, *Biotechniques* 11:152-154, 156 (1991)). Because the light chain amino terminal sequences were known, a specific primer corresponding to the likely 5' sense
20 sequence was used (5' ACTAGTCGACATGAGTGTGCTCACTCAGGTCCTGG (GC)GTTG 3' (SEQ ID NO:24); Jones, S.T., and Bendig, M.M., *Bio/Technology* 9:88-89 (Erratum) (1991)) for cDNA amplification. For cloning of the heavy chain, mouse heavy chain variable primers 1-12 were used as described (Jones, S.T., and Bendig, M.M., *Bio/Technology* 9:88-89 (Erratum) (1991)). All heavy
25 chains amplified best with primer 9; though lesser amplification was also seen with primers 12, 10 and 6. The PCR products were isolated by low melt agarose fractionation and ligated into a vector. The light chain PCR product was ligated into PCR II vector (Invitrogen, San Diego, CA) The heavy chain PCR product from primer 9 was ligated into PCR II.1 vector (Invitrogen, San Diego, CA).
30 After transformation, the plasmid DNA was isolated and subjected to restriction

digestion with EcoR1. Two clones from each heavy and light chain were expanded and the DNA harvested. Both strands of the cDNA clones were sequenced using T7 and M13 primers with an ABI Prism automated sequencing apparatus. The cDNA sequences and deduced amino acid sequences are shown in Figures 11-16 (SEQ ID NOS:4-15).

Example 4

Preparation and Characterization of Chimeric and Humanized Antibodies

In designing the sequence for a chimeric or humanized antibody, there are many parameters to consider. In the constant regions, a whole antibody may be made, or an antibody fragment (Fab and Fab'2) can be made. The constant regions may be murine or human. It is an accepted practice to replace murine constant regions with human constant regions, thus forming a "chimeric" antibody. Chimeric antibodies are less immunogenic than murine antibodies and are thus more acceptable in the clinic.

The subclass of the antibody must also be considered. It is most common to express recombinant antibodies as IgGs, but within this class, one must choose amongst recombinant chimeric human IgG1, IgG2, IgG3, and IgG4. These subclasses have different biological properties. The present inventors took a conservative approach of using IgG2 because 1) the strong complement activating properties of IgG1 and IgG3 were not needed for this antibody and 2) IgG2 may be more straightforward to manufacture than IgG4. Any of the other subclasses could be made with the same specificity following similar strategies.

There are also parameters to consider in designing the variable region. The antibodies could be constructed to be chimeric or humanized. The chimeric antibody (murine V region, human constant region) is a more conservative approach, and virtually guarantees very similar antigen-binding activity to the murine antibody. With humanization, there is the risk of reducing the affinity

and/or biological activity of the antibody, but it can be presumed that the antibody will be less immunogenic. The present inventors have produced chimeric antibody as well as three forms of the humanized antibody.

5 Depending upon the strategy taken, humanization of any particular antibody can result in many different variable regions. At the simplest level, humanization consists of choosing a human variable region to serve as a template, and then deciding which residues should be "human" and which "murine". Thus, the choice of both the human template and which residues to maintain as human will affect the final sequence.

10 In general, the strategy the present inventors have taken is to choose from among the human germline variable region genes for the templates. Alternatively, one can choose from rearranged variable region genes, both those which have and have not undergone somatic mutation. The rationale for the first strategy is that somatic mutations can introduce immunogenic epitopes, while germline genes
15 would have less potential for doing so. The selection was further limited to germline genes which are known to be rearranged and expressed as functional proteins in humans.

20 The choice of which germline gene to use as template is governed by the overall sequence similarity between the murine sequence and the human sequence; the structural similarities between the two sequences (Chothia and Lesk, *J. Mol. Biol.* 196:901 (1987)); the anticipated ability of the chosen heavy chain template to pair with the chosen light chain template; and the presence of the germline gene in the majority of humans. The choice of which residues should be murine is governed by which residues are thought to come in contact with antigen and
25 which are necessary to maintain the positioning and orientation of those residues which might contact antigen.

30 Variable regions were assembled from oligonucleotides and inserted into expression vectors containing the human gamma 2 constant region (for the VH region) and human kappa constant region (for the VL region). Heavy and light chain vectors were verified by nucleotide sequence and ability to direct the

synthesis of antigen binding immunoglobulin (Ig) in COS cells (transient expression). Selected heavy and light chain vectors were then cotransfected into CHO cells to produce stable cell lines expressing the chimeric and humanized antibodies. Antibody was purified and tested for activity by antigen binding ELISA, ability to block the inhibitory activity of α 2-AP in a plasmin assay, and ability to facilitate lysis of human clots by urokinase.

A. Construction of Chimeric and Humanized Antibody Vectors

A functional light chain variable region is formed by the rearrangement and juxtaposition of a V gene segment and J gene segment. Therefore, it was necessary to find the best match for each of these segments and combine them to form a human template. A FASTA search (using the Wisconsin Package Interface) of amino acids 1-95 (Kabat numbering system; V gene proper) of murine 77A3 (m77A3) light chain against a database of human Vk germline genes showed that m77A3 is clearly most similar to the human VkI subgroup (69.2% - 71.6% identity vs less than 60% identity to sequences outside this subgroup). From among the Vk I sequences, the sequence with GenBank accession # X59312 (also known as the O2/O12 gene) was chosen as a likely candidate because of the match with structurally important positions and because of its prevalent expression in humans. The human template for the light chain was completed by the addition of the human Jk2 sequence. This J region was chosen because of its high degree of similarity with the murine J region of 77A3.

A functional heavy chain variable region is formed by the rearrangement and juxtaposition of a V gene segment, a D gene segment, and a J gene segment. Therefore, it was necessary to find the best match for each of these segments and combine them to form a human template. A FASTA search (using the Wisconsin Package Interface) of amino acids 1-94 (Kabat numbering system; V gene proper) of murine 77A3 heavy chain against a database of human VH germline genes showed that m77A3 is clearly most similar to the human VH7 family (77 %

identity) with the human VH1 family having the next best match (about 60 % identity). The human VH7 family is mostly composed of pseudogenes; the only active gene (7-04.1, Accession # X62110) is polymorphic in the human population (i.e. not all people have it) and therefore, in some people, this V gene could be more immunogenic than others. As an alternative human template for the heavy chain, the V gene with accession number Z12316 (1-18 gene) was chosen. This sequence is very similar to 7-04.1 except for the H2 loop and FR3 region. A human template for the D region was not considered because this region lies entirely within the H3 loop, the sequence of which is generally pivotal for antigen binding and therefore likely to entirely follow the murine sequence in a humanized antibody. The human template for the heavy chain was completed by the addition of the human JH5 sequence. This J region was chosen because of its high degree of similarity with the murine J region of 77A3.

Following the selection of human templates for the heavy and light chain variable regions, it was necessary to determine which positions should follow the murine sequence vs which positions should follow the human sequence. The following criteria were used in selecting positions to follow the murine sequence: all positions falling within the CDR loops; all positions known to influence the conformation and/or spatial position of CDR loops (so called structural determinants; Chothia and Lesk, *J. Mol. Biol.* 196:901 (1987), Lesk and Tramontano, *in: Antibody Engineering*, W.H. Freeman and Co., pp.7-38 (1992)); residues which were close enough to interact with residues in the CDR loops; and residues at or proximal to the VH-VL domain interface. All other residues followed the human sequence. These items are discussed in greater detail below.

Positions falling within the CDR loops are shown enclosed within the boxes with solid borders and structural determinants are marked with an * in the row below the position number in Figures 17-19.

In order to determine which residues were close enough to interact with the CDR loops, it was necessary to generate an approximate molecular model of the Fv region of murine 77A3. The molecular model was built based on the

combined variable light chain of an anti-lysozyme mAb (D1.3) and the variable heavy chain of an anti-neuraminidase mAb (1ncca) as structural template. CDR loop sequences were assigned to canonical loop conformations and a possible conformation for CDR H3 was extracted from the Protein Data Bank. The modeling building protocol followed procedures described by Bajorath & Novotny (*Therapeutic Immunol.* 2:95-105 (1995)). Likewise, residues at or proximal to the VH-VL domain interface were identified and the murine residues were used for the humanized antibody. In all, for h77A3-1 heavy chain, h77A3-2 heavy chain, and for the common light chain there were 7, 18, and 11 murine residues, respectively, used outside of the CDR loops.

In order to prepare vectors encoding these chains, the amino acid sequence must be back translated into nucleotide sequence. For the most part, this was done simply by using the nucleotide sequence from the human template in cases where the amino acid residue is derived specifically from the human template; otherwise, the nucleotides from the murine sequence were used. At a few positions, silent substitutions were made in order to eliminate restriction sites.

Finally, signal peptides must be added to the sequence. For both the chimeric and humanized light chains, signal peptides corresponding to that of the murine 77A3 light chain were used. For the chimeric and humanized heavy chains, the same signal peptide as for the light chains was used. Alternatively, signal peptides corresponding to that of murine 77A3 VH or any other signal peptide can be used in the chimeric and humanized heavy chains.

Two humanized antibodies were created: h77A3-1 and h77A3-2. A third version of the humanized heavy chain was created by including an oligonucleotide designed for h77A3-1 in the construction of h77A3-2. This resulted in a hybrid molecule that was identical to h77A3-2 except for amino acids Ser and Leu at positions 9 and 11 of the heavy chain. One chimeric antibody, c77A3, was generated.

Amino acid and nucleotide sequences of h77A3-1 and h77A3-2 heavy and light chains are shown in Figures 17-19 (SEQ ID NOS:16-21). The common light

chain is shown in Figure 17 (mature protein is amino acid residues 1 to 107 of SEQ ID NO:17). The heavy chain of h77A3-1 is shown in Figure 18 (mature protein is amino acid residues 1 to 119 of SEQ ID NO:19). The heavy chain of h77A3-2 is shown in Figure 19 (mature protein is amino acid residues 1 to 123 of SEQ ID NO:21).

Expression vectors for chimeric and humanized 77A3 light and heavy chains were prepared in three stages: (1) construction of cassettes containing human light or heavy chain constant region genes (pD16-hCka and pD20-hy2a, respectively); (2) preparation of a PCR product containing the light or heavy chain variable region; and (3) insertion of the variable region into the appropriate expression cassette.

Plasmid pD13 was constructed and derived from the pcDNA3 plasmid (Invitrogen) in two steps. The SV40 promoter/enhancer and neomycin resistance genes were removed from pcDNA3 by digestion with NaeI and isolation of the 3.82 kb fragment. These genes were replaced by the SV40 promoter/enhancer and dhfr gene from pSV2-dhfr. The DNA containing the pSV2-dhfr sequence was isolated as a 1.93 kb fragment after digestion with PvuII and BamHI. The 3.82 and 1.93 kb fragments were ligated together and used to transform MC1061 bacteria following filling in the protruding ends of the 1.93 kb fragment from pSV2-dhfr. The correct product (designated pD12) was confirmed by the release of an 890 bp fragment following HindIII digestion.

The polylinker was replaced with alternative restriction sites by digesting the resultant vector above with Asp718 and Bsp120I. The following oligonucleotides were annealed to the vector and cloned by ExoIII cloning (K. Hsiao, *Nucl. Acid. Res.* 21:5528-5529 (1993)) to complete the plasmid pD13:

5' TAGGGAGACCCAAGCTTGGTACCAATTTAAATTGATATCTCCTT AG
GTCTCGAGTCTCTAGATAACCGGTCAATCGATTGGGATTCTT 3' (SEQ
ID NO:25) and

5' GACACTATAGAATAGGGCCCTTCCGCGGTTGGATCCAACACGT
GAAGCTAGCAAGCGGCCGCAAGAATTCCAATCGATTGACCGGTTA 3'

(SEQ ID NO:26). The resulting plasmid was used to transform competent *E. coli* DH5 α and the correct product was confirmed by sequencing the polylinker region.

5 Plasmid pD16 was derived from the pcDNA3 plasmid (Invitrogen) in a series of steps which: add a polylinker sequence upstream of the CMV promoter for linearization; delete the SV40 promoter/enhancer and neomycin resistance gene and replace them with the histone H3 transcription termination sequence, the SV40 promoter (enhancer deleted) and DHFR gene; and insert the gastrin transcription termination sequence upstream of the CMV promoter.

10 pcDNA3 (Invitrogen) was digested with BglII and annealed to the following oligonucleotides:

5' primer: 5'-GATCTGCTAGCCCGGGTGACCTGAGGCGCGCCTTTG
GCGCC-3' (SEQ ID NO:27);and

15 3' primer: 3'-ACGATCGGGCCCCACTGGACGCCGCGCGGAAACCGCGG
CTAG-5' (SEQ ID NO:28).

The plasmid was then ligated. After ligation, the resulting plasmid (pcDNA3-LSI) was used to transform competent *E. coli* DH5 α and the correct construct was confirmed by release of a 230 bp fragment following restriction enzyme digestion with NheI and NruI.

20 Plasmid pcDNA3-LSI was then digested with NgoMI, PvuI and BsmI. Following digestion, a 2.0 kb NgoMI-PvuI fragment was isolated. Plasmid pD12 (described above) was digested with PvuI and SphI to remove the SV40 enhancer and a 3.6 kb fragment was isolated. The following oligonucleotides, encoding the histone H3 transcription termination sequence were annealed and then ligated with
25 the 2.0 kb NgoMI-PvuI fragment and 3.6 kb PvuI-SphI fragment:

5' primer: 5'-CCGGGCCTCTCAAAAAAGGGAAAAAAGCATG-3' (SEQ ID
NO:29); and

3' primer: 3'-CGGAGAGTTTTTCCCTTTTTTTC-5' (SEQ ID NO:30).

The resulting plasmid pcTwD-LS1 was confirmed by the production of 3.3, 0.95, 0.82 and 0.63 kb fragments after digestion with NheI plus NciI and the production of 4.2, 1.0, 0.26 and 0.23 kb fragments after digestion with SphI plus BstEII.

Insertion of the gastrin transcription termination sequence to form plasmid pD16 was accomplished by digesting pcTwD-LS1 with BssHII and NarI and isolating the 5.7 kb fragment and ligating with the following annealed oligonucleotides:

5' primer: 5'-CGCGCCGGCTTCGAATAGCCAGAGTAACCTTTTTTTTTTAA
TTTTATTTTATTTTATTTTGTGAGATGGAGTTTGG-3' (SEQ ID NO:31); and
3' primer: 3'-GGCCGAAGCTTATCGGTCTCATTGGAAAAAAAAAATTAAAT
AAAAT AAAATAAAAACTCTACCTCAAACCGC-5' (SEQ ID NO:32).

After ligation, the product was used to transform competent *E. coli* MC1061 and the correct construction was confirmed by the production of 4.8, 0.66 and 0.31 kb fragments after digestion with NgoMI plus SpeI and the production of 3.3, 1.0, 0.82 and 0.67kb fragments following digestion with NgoMI plus NcoI.

Plasmid pD17 was derived from pD16 by the removal of the NheI site from the linearization polylinker. This was accomplished by digestion of pD16 with BstII and NheI and filling the protruding ends using Klenow polymerase. The reaction mixture was self-ligated and used to transform competent *E. coli* DH5 α .

pD17 was digested with Asp718I and Bsp120I to remove a polylinker which was replaced by the 113 bp Asp718I/Bsp120I polylinker from pD13. After ligation, the resulting intermediate plasmid pD20 had the NheI site required for inserting heavy chain V genes. pD20 was distinguished from pD17 by linearization with NheI, and distinguished from pD13 by linearization with BssH II which cuts only once within the linearization site polylinkers of pD16, pD17 and pD20. Finally, DNA sequencing was used to confirm the polylinker in pD20.

A 2.9 kb EcoRI fragment was isolated from pGk.11 (Walls *et al.*, *Nucl. Acid. Res.* 21:2921-2929 (1993)) and this was ligated into the plasmid pD13 (described above) previously digested with EcoRI. This construct (pD13-hCka) containing the human Ck exon and flanking intron sequences was used to transform

E. coli DH5 α and the correct product was confirmed by restriction digestion. Digestion with EcoRI resulted in fragments of 5.7, 2.8 and 0.3 kb and digestion with SacI resulted in fragments of 7.1, 1.1 and 0.5 kb.

Construction of the light chain expression cassette was completed by removing the C κ fragment along with the flanking polylinker sequences from pD13 and inserting it into pD16. Plasmid pD13-hC κ a was digested with Asp718I and Bsp120I to release the C κ fragment and polylinker sequences. The same enzymes were used to linearize pD16 and the C κ containing fragment was ligated into pD16 to form pD16-hC κ a. Following transformation of DH5 α *E. coli* and amplification, the correct construct was confirmed by the release of 2.9 kb fragment following digestion with Asp718I and Bsp120I and linearization following digestion with a restriction enzyme present in pD16, but not pD13. The nucleotide sequence was also confirmed by sequencing various regions of the construct.

A genomic DNA fragment encoding the human γ 2 gene was preassembled in pIC, and then transferred into pD20 as follows. Phage clone Phage 5A (Ellison and Hood, *Proc. Natl. Acad. Sci.*, 79: 1984-1988 (1982)), containing the human γ 2 gene was digested with HindIII and cloned into the HindIII site of pUC18 to form the vector p γ 2. In p γ 2, the 5' end of the γ 2 gene is adjacent to the polylinker region.

pG was derived from pSV2-gpt by digestion with Hind III and Bgl II, Klenow fill in, and religation. This served to remove a 121 bp Hind III-Bgl II fragment. p γ 2 was then digested with BamH I and inserted into the BamH I site of pG to form pG γ 2.2. pG γ 2.2 contains a BglII site 3' of the coding region that would interfere with later cloning steps. To remove this restriction site, pG γ 2.2 was first digested with Bgl II, the sticky ends filled in by Klenow DNA polymerase I, then the plasmid religated. The resulting intermediate plasmid, pG γ 2.3 was screened for lack of digestibility with Bgl II.

For purposes of later cloning in variable region genes, it was important to provide a restriction site in the γ 2 containing cassette. This is conveniently done by mutating the nucleotides encoding the first two amino acids of the CH1 exon to

encode an Nhe I site (Coloma M.J. *et al*, *J. Immunological Methods* 152:89-104(1992)). Previously, an Nhe I to Bst E II fragment from the human $\gamma 4$ gene was cloned. In this region, human $\gamma 2$ and human $\gamma 4$ genes encode identical amino acids. Thus, the $\gamma 4$ containing vector (pIChy4.1) could serve as a source for the 5' end of the $\gamma 2$ gene. This vector was obtained as follows: The 8.6 kb BamH I fragment from Phage 5D (Ellison, J. *et al DNA* 1:11-18 (1981)), containing the human $\gamma 4$ gene, was subcloned into pUC, resulting in the plasmid pUChy4. pUChy4 served as the template for a PCR reaction involving the following primers: sense primer: 5'-ATCGATGCTAGCACCAAGGGCCCA-3' (SEQ ID NO:33); and antisense primer: 5'-CTCGAGGGGGTCCACCACGCTGCTGA-3' (SEQ ID NO:34). The sense primer contained a ClaI site for subcloning the PCR product into pIC20R (Marsh J.L., *et al, Gene* 32: 481-485 (1984)) adjacent to a synthetic NheI site (underlined). Note that the bases for the NheI site can encode the first two amino acids (Alanine and Serine) for the human $\gamma 1$, $\gamma 2$, $\gamma 3$ or $\gamma 4$ CH1 exon. The antisense primer has an Xho I site for subcloning into pIC20R, next to a BstE II site (underlined) which is in the CH1 exon of the human $\gamma 4$ and $\gamma 2$ gene. The PCR product formed was restricted with Cla I + Xho I then ligated into pIC20R which had been digested by the same enzymes, to generate the intermediate pIChy4.1.

pGy2.3 was digested with BamH I and Hind III and a 6.1 Kb fragment including the human $\gamma 2$ gene locus was isolated from a 1.4% agarose gel for purification by the QiaexTM gel extraction kit (Qiagen, Chatsworth, CA). The 2.9 Kb pIChy4.1 plasmid was treated in a similar manner, and the two fragments were ligated together to form the intermediate vector pIChy2.1. To screen, an EcoR I digest yielded appropriate fragment sizes of 6.3 Kb and 2.6 Kb.

pIChy2.1 contained a duplication of the 5' portion of the human $\gamma 2/\gamma 4$ CH1 exon. In order to remove the duplicated region, it was digested with BstE II giving fragment sizes of 4.0 Kb, 1.8 Kb, 1.6 Kb, 1.1 Kb, and 0.4 Kb. The 4.0 Kb fragment was isolated from a 1.4% agarose gel, while the 1.6 Kb fragment was separated and isolated away from the 1.8 Kb fragment in 4% NuSieveTM GTG (FMC Bioproducts, Rockland, ME) agarose. Both fragments were purified by Qiagen gel extraction

prior to ligating them together to prepare pIChy2.2. In order to confirm the proper orientation of the two fragments the following primers were used to determine that the 3' portion of the human $\gamma 4$ CH1 exon's BstE II sticky end had joined with the 5' end of the human $\gamma 2$ CH1 exon (thus forming a contiguous human $\gamma 2$ locus in pIC20R):

sense primer: 5'-AACAGCTATGACCATGATTAC-3' (SEQ ID NO:35); and
antisense primer: 5'-CACCCAGCCTGTGCCTGCCTG-3' (SEQ ID NO:36).

The sense primer is homologous to sequence 5' of the pIC20R EcoR I site that is adjacent to the Cla I site. The antisense primer was chosen to be 500 bp downstream of the sense strand primer, and is homologous to sequence within the human $\gamma 2$ CH1 to CH2 intron. Thus, visualization of a 500 bp PCR product in a 1.4% agarose gel confirmed that the hybrid human $\gamma 4$ - $\gamma 2$ CH1 exon formed and was oriented in a contiguous manner to the remainder of the locus. pIChy2.2 was digested with EcoR I to give the expected 2.6 Kb and 1.9 Kb fragments. The entire human $\gamma 2$ CH1 exon was confirmed by DNA sequencing.

The 1.8 Kb Nhe I + Hind III fragment containing the human $\gamma 2$ gene locus was removed from pIChy2.2 for ligation into plasmid pD20 opened by Nhe I + Hind III. The resulting vector is the expression cassette pD20-hy2a.

The variable region (V) genes for both chimeric and humanized antibodies were synthesized by a modification of the non template specific PCR protocol (Prodromou C., and Pearl L. H., *Protein Eng.* 5: 827-829 (1992)). The PCR products included DNA encoding both the signal peptide and variable region proper as well as flanking sequences to facilitate insertion into the vector as well as correct splicing (light chain only).

The following primers were used:

LH1, sense chimeric 77A3 VH outer primer (30mer), 5'-CGATTGGAATTCTTG CGGCCGCTTGCTAGC-3' (SEQ ID NO:37);

LH2, sense chimeric 77A3 VH primer 1 (80 mer), 5'-CTTGCGGCCGCTTGCTA
GCATGGATTGGGTGTGGAACCTTGCTATTCCTGATGGCAGCTGCCCAA
AGTATCCAAGCACAGA-3' (SEQ ID NO:38);

5 LH3, anti-sense chimeric 77A3 VH primer 2 (80 mer), 5'-CTTGACTGTTTC
TCCAGGCTTCTTCAGCTCAGGTCCAGACTGCACCAACTGGATCTGTGC
TTGGATACTTTGGGCAGCTG-3' (SEQ ID NO:39);

LH4, sense chimeric 77A3 VH primer 3 (80 mer), 5'-CTGAAGAAGCCT
GGAGAAACAGTCAAGATCTCCTGCAAGGCTTCTGGGTATACCTTCAC
AACTATGGAATGAACTGGGT-3' (SEQ ID NO:40);

10 LH5, anti-sense chimeric 77A3 VH primer 4 (80 mer), 5'-TCTTGGTGTTTAT
CCAGCCCATCCACTTTAAACCCTTTCCTGGAGCCTGCTTCACCCAGTT
CATTCCATAGTTTGTGAAG-3' (SEQ ID NO:41);

15 LH6, sense chimeric 77A3 VH primer 5 (80 mer), 5'-AGTGGATGGGCT
GGATAAACACCAAGAGTGGAGAGCCAACATATGCTGAAGAGTTCAA
GGGACGGTTTGCCTTCTCTTTG-3' (SEQ ID NO:42);

LH7, anti-sense chimeric 77A3 VH primer 6 (80 mer), 5'-TCCTCATTTTTGA
GGTTCTTGATCTGCAAATTGGCAGTGCTGGCAGAGGTTTCCAAAGAG
AAGGCAAACCGTCCCTTGAA-3' (SEQ ID NO:43);

20 LH8, sense chimeric 77A3 VH primer 7 (80 mer), 5'-GCAGATCAAGAACC
TCAAAAATGAGGACACGGCTACATATTTCTGTGCAAGATGGGTACCT
GGGACCTATGCCATGGACT-3' (SEQ ID NO:44);

LH9, anti-sense chimeric 77A3 VH primer 8 (80 mer), 5'-TGGGCCCTTGGTGC
TAGCTGAGGAGACGGTGACTGAGGTTCCTTGACCCAGTAGTCCATG
GCATAGGTCCCAGGTACCC-3' (SEQ ID NO:45);

25 LH10, anti-sense murine 77A3 VH outer primer (29 mer),
5'-GGGAAGACGGATG GGCCCTTGGTGCTAGC-3' (SEQ ID NO:46);

LH11, sense chimeric 77A3 VL outer primer (30mer), 5'-ATTTAAATTGAT
ATCTCCTTAGGTCTCGAG-3' (SEQ ID NO:47);

LH12, sense chimeric 77A3 VL primer 1 (79 mer), 5'-ATTTAAATTGATATCTCC
TTAGGTCTCGAGATGAGTGTGCTCACTCAGGTCCTGGCGTTGCTGCT
GCTGTGGCTTACAG-3' (SEQ ID NO:48);

5 LH13, anti-sense chimeric 77A3 VL primer 2 (78 mer), 5'-AGATGCAGATAGG
GAGGCTGGAGACTGAGTCATCTGGATGTCACATCTGGCACCTGTAAG
CCACAGCAGCAGCAACGC-3' (SEQ ID NO:49);

LH14, sense chimeric 77A3 VL primer 3 (78 mer), 5'-GTCTCCAGCCTCCCTA
TCTGCATCTGTGGGAGAACTGTCACCATCACATGTCGAGCAAGTGG
GAATATTCACAATTA-3' (SEQ ID NO:50);

10 LH15, anti-sense chimeric 77A3 VL primer 4 (78 mer), 5'-TATAGACCAG
GAGCTGAGGAGATTTTCCCTGTTTCTGCTGATACCATGCTAAATAATT
GTGAATATTCCCCTTGCTC-3' (SEQ ID NO:51);

LH16, sense chimeric 77A3 VL primer 5 (78 mer), 5-AAATCTCCTCAGCT
CCTGGTCTATAATGCAAAAACCTTAGCAGATGGTGTGCCATCAAGGT
15 TCAGTGGCAGTGGATCA-3' (SEQ ID NO:52);

LH17, anti-sense chimeric 77A3 VL primer 6 (78 mer); 5'-CTCCCCAAAATCT
TCAGGCTGCAGGCTGTTGATCCTGAGAGAAAATTGTGTTCCCTGATCC
ACTGCCACTGAACCTTGAT-3' (SEQ ID NO:53);

20 LH18, sense chimeric 77A3 VL primer 7 (78 mer),
5'-GCCTGCAGCCTGAAGATTTTGGGAGTCATTACTGTCAACATTTTG
GACCACTCCGTGGACGTTTCGGTGGAGGCACCA-3' (SEQ ID NO:54);

LH19, anti-sense chimeric 77A3 VL primer 8 (81 mer), 5'-TTCCAATCGATTGA
CCGGTTATCTAGAGACTCGAGACTTACGTTTGATTTCAGCTTGGTGC
CTCCACCGAACGTCCACGG-3' (SEQ ID NO:55);

25 LH20, anti-sense chimeric 77A3 VL outer primer (30mer), 5'-TCGATTGA
CCGGTTATCTAGAGACTCGAGA-3' (SEQ ID NO:56);

LH21, anti-sense humanized 77A3 VL primer 2 (78 mer), 5'-AGATGCAGATA
GGGAGGATGGAGACTGAGTCATCTGGATGTCACATCTGGCACCTGTA
AGCCACAGCAGCAGCAACGC-3' (SEQ ID NO:69)

LH22, sense humanized 77A3 VL primer 3 (78 mer), 5'-GTCTCCATCCTCC
CTATCTGCATCTGTGGGAGACAGAGTCACCATCACATGTCGAGCAAG
TGGGAATATTCACAATTA -3' (SEQ ID NO:70)

5 LH23, sense humanized 77A3 VL primer 5 (78 mer), 5'-AAATCTCCTCAA
CTCCTGGTCTATAATGCAAAAACCTTAGCAAGTGGTGTGCCATCAAG
GTTCAGTGGCAGTGGATCA -3' (SEQ ID NO:71)

LH24, anit-sense humanized 77A3 VL primer 6 (78 mer), 5'-CTCCCAAATC
TTCAGGCTGCAGGCTGCTGATGGTGAGAGTAAAATCTGTTCCTGATC
CACTGCCACTGAACCTTGAT -3' (SEQ ID NO:72)

10 LH25, sense humanized 77A3 VH -1 primer 1 (80 mer), 5'-CTTGCGGCCGCTTG
CTAGCATGAGTGTGCTCACTCAGGTCCTGGCGTTGCTGCTGCTGTGG
CTTACAGGTGCCAGATGTC -3' (SEQ ID NO:57);

15 LH26, anti-sense humanized 77A3 VH -1 primer 2 (80 mer); 5'-GACTGAGGCT
CCAGGCTTCTTCAGCTCAGATCCAGACTGCACCAACTGGATCTGACA
TCTGGCACCTGTAAGCCACAGCA -3' (SEQ ID NO: 58);

LH27, sense humanized 77A3 VH -1 primer 3 (80 mer), 5'-GAGCTGAAGAAGC
CTGGAGCCTCAGTCAAGATCTCCTGCAAGGCTTCTGGGTATACCTTCA
CAAATATGGAATGAACTG -3' (SEQ ID NO:59);

20 LH28, anti-sense humanized 77A3 VH -1 primer 4 (80 mer) 5'-TGGTGTTCATC
CAGCCCATCCACTCTAAACCTTGTCCTGGAGCCTGTCGCACCCAGTTC
ATTCCATAGTTTGTGAAGGTA -3' (SEQ ID NO:60);

LH29, sense humanized 77A3 VH -1 primer 5 (80 mer), 5'-TAGAGTGGATGGG
CTGGATAAACACCAAGAGTGGAGAGCCAACATATGCTGAAGAGTTCA
AGGGACGGTTTGTCTTCTCT -3' (SEQ ID NO:61);

25 LH30, anti-sense humanized 77A3 VH -1 primer 6 (80 mer), 5'-TCAGCTTTGAGG
CTGCTGATCTGCAAATAGGCAGTGCTGACAGAGGTGTCCAAAGAGAA
GACA AACCGTCCCTTGAATC -3' (SEQ ID NO:62);

30 LH31, sense humanized 77A3 VH -1 primer 7 (80 mer), 5'-TTTGCAGATCAG
CAGCCTCAAAGCTGAGGACACGGCTGTGTATTTCTGTGCAAGATGGG
TACCTGGGACCTATGCCATGG -3' (SEQ ID NO:63);

LH32, anti-sense humanized 77A3 VH -1 primer 8 (80 mer), 5'-GCCCTTGGTG
CTAGCTGAGGAGACGGTGACCGTGGTTCCTTGACCCAGTAGTCCAT
GGCATAGGTCCCAGGTACCCATC -3' (SEQ ID NO:64);

5 LH33, anti-sense humanized 77A3 VH -2 primer 2 (80 mer), 5'-TGCTGTGGCT
TACAGGTGCCAGATGTCAGATCCAGTTGGTGCAGTCTGGAGCTGAGG
TGAAGAAGCCTGGAGCCTCAGTC -3' (SEQ ID NO:65);

LH34, sense humanized 77A3 VH -2 primer 5 (80 mer), 5'-TAGAGTGGATGGGC
TGGATAAACACCAAGAGTGGAGAGCCAACATATGCTGAAGAGTTCAA
GGGACGGTTTACCTTCACC -3' (SEQ ID NO:66);

10 LH35, anti-sense humanized 77A3 VH -2 primer 6 (80 mer), 5'-TCAGATCTGAG
GCTCCTGATCTCCAAATAGGCAGTGCTCGTAGAGGTGTCCAAGGTGA
AGGTAAACCGTCCCTTGAAGTC -3' (SEQ ID NO:67); and

15 LH36, sense humanized 77A3 VH -2 primer 7 (80 mer), 5'-TTTGGAGATC
AGGAGCCTCAGATCTGACGACACGGCTGTGTATTTCTGTGCAAGATG
GGTACCTGGGACCTATGCCATGG -3' (SEQ ID NO:68).

Table 3 summarizes how the above primers were used in the non-template
PCR protocol.

Table 3. Use of primers in non-template PCR

	Heavy chains			Light chains	
	chimeric	humanized-1	humanized-2	chimeric	humanized
outer primer (sense)	LH1 (SEQ ID NO:37)	LH1 (SEQ ID NO:37)	LH1 (SEQ ID NO:37)	LH11 (SEQ ID NO:47)	LH11 (SEQ ID NO:47)
V1 (sense)	LH2 (SEQ ID NO:38)	LH25 (SEQ ID NO:57)	LH25 (SEQ ID NO:57)	LH12 (SEQ ID NO:48)	LH12 (SEQ ID NO:48)
V2 (antisense)	LH3 (SEQ ID NO:39)	LH26 (SEQ ID NO:58)	LH33 (SEQ ID NO:65)	LH13 (SEQ ID NO:49)	LH21 (SEQ ID NO:69)
V3 (sense)	LH4 (SEQ ID NO:40)	LH27 (SEQ ID NO:59)	LH27 (SEQ ID NO:59)	LH14 (SEQ ID NO:50)	LH22 (SEQ ID NO:70)
V4 (antisense)	LH5 (SEQ ID NO:41)	LH28 (SEQ ID NO:60)	LH28 (SEQ ID NO:60)	LH15 (SEQ ID NO:51)	LH15 (SEQ ID NO:51)
V5 (sense)	LH6 (SEQ ID NO:42)	LH29 (SEQ ID NO:61)	LH34 (SEQ ID NO:66)	LH16 (SEQ ID NO:52)	LH23 (SEQ ID NO:71)
V6 (antisense)	LH7 (SEQ ID NO:43)	LH30 (SEQ ID NO:62)	LH35 (SEQ ID NO:67)	LH17 (SEQ ID NO:53)	LH24 (SEQ ID NO:72)
V7 (sense)	LH8 (SEQ ID NO:44)	LH31 (SEQ ID NO:63)	LH36 (SEQ ID NO:68)	LH18 (SEQ ID NO:54)	LH18 (SEQ ID NO:54)
V8 (antisense)	LH9 (SEQ ID NO:45)	LH32 (SEQ ID NO:64)	LH32 (SEQ ID NO:64)	LH19 (SEQ ID NO:55)	LH19 (SEQ ID NO:55)
outer primer (antisense)	LH10 (SEQ ID NO:46)	LH10 (SEQ ID NO:46)	LH10 (SEQ ID NO:46)	LH20 (SEQ ID NO:56)	LH20 (SEQ ID NO:56)

Briefly, 8 adjacent oligonucleotides which represent a synthetic light or heavy chain V gene are synthesized (V1-V8 in Table 3). Four sense strand oligonucleotides alternate with 4 overlapping antisense strand oligonucleotides of 78-81 nt in length. These PCR primers overlap each other by 24-27 nt. Note that for oligonucleotide primers V1 and V8 (Table 3), their 5' end is designed to overlap with 15 -30 nt of the vector sequence, while their 3' end overlaps 48-65 nt of the signal peptide (V1) or the V gene sequence (V8). The 8 oligonucleotide primers

are all included in the same first round PCR. Reaction conditions for this 1st round PCR were 0.125 picomoles of each primer, 10 μ l of 10X Pfu buffer (Stratagene Inc., San Diego, CA), 10 nanomoles dNTP's (Boehringer Mannheim, Indianapolis, IN), 10% dimethylsulfoxide (DMSO), and 2.5 units cloned Pfu DNA polymerase I (Stratagene Inc., San Diego, CA) in a 100 μ l reaction volume. Reactants were first denatured at 95°C for 5 min, annealed at 45 °C for 5 min, and extended at 72 °C for 1 min, followed by 25 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec. The 25 cycles were followed by a final extension at 72 °C for 7 min in a Perkin-Elmer DNA Thermal Cycler (Norwalk, CT).

The amplified PCR product was electrophoresed through a 1.4% agarose gel and the smear of DNA running between approximately 350 bp - 500 bp was cut out prior to purification by the Qiaex™ II gel extraction kit (Qiagen, Chatsworth, CA). This purified non template specific PCR product served as the template for a 2nd round PCR. To complete the 2nd round PCR, two additional outer primers are utilized. These outer primers are homologous to 29 - 30 nt of the vector sequence that is either 5' (sense primer) or 3' (antisense primer) of the linearized cloning site within the mammalian expression cassette vector. This allowed for the amplified PCR product to be subcloned into the vector by bacterial homologous recombination (Jones, D.H. and Howard, B. H., *BioTechniques* 10: 62-66 (1991)). Thus, the reaction conditions for the 2nd round PCR were 0.125 picomoles each of outer sense and antisense primers, 10 μ l of 10X Pfu buffer, 10 nanomoles dNTP's, 10% DMSO, 2.5 units Pfu DNA polymerase I, and approximately 100 ng of 1st round PCR template DNA. The reactants underwent the same thermocycle program described above. Subsequently, the amplicand from this reaction was removed from a 1.4% agarose gel and purified with the Qiagen™ II gel extraction kit.

200ng -1000ng of PCR product was mixed with an equal weight of linearized vector, and this mixture was used to transform 200 ml of competent *E. coli* DH5 α cells (GIBCO BRL/ Life Technologies, Gaithersburg, MD). Transformed cells were selected by 100 μ g/ml ampicillin in LB agarose. Typically,

pD16-hCka digested with Xho I was used for subcloning light chain V genes. pD20-hg2a digested with Nhe I served as the vehicle for heavy chain V gene constructs.

5 In order to confirm that the V gene of interest had been inserted into the expression vector, two screens were performed. The primary screen was by PCR, while the secondary screen was by restriction digest. Each individual colony of bacteria was picked into 5 ml of T broth (GIBCO BRL/ Life Technologies, Gaithersburg, MD) containing 100 μ g/ml ampicillin and grown 8 - 16 hr at 37 °C with shaking. The conditions for the PCR screen were 0.125 picomoles of both
10 outer primers (Table 3), 2 ml 10X Mg⁺² buffer (Boehringer Mannheim, Indianapolis, IN), 10 nanomoles dNTP's (Boehringer Mannheim, Indianapolis, IN), 1 unit Taq DNA polymerase I (Boehringer Mannheim, Indianapolis, IN), and 1 μ l of the liquid culture growth (which served as the source of DNA template since the cells lysed at high temperature) in a 20 μ l volume. Reactants first underwent denaturation at
15 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 25 sec, annealing at 45 °C for 25 sec, and extension at 72 °C for 12 sec. The cycles were followed by a final extension at 72 °C for 7 min. Positives were determined by size comparison relative to a DNA standard marker after electrophoresis through a 1.4% agarose gel.

20 For the secondary screen, midi DNA preparations (Qiagen, Chatsworth, CA) were made from bacterial pellets and a portion was digested with either Xho I (VL genes) or Nhe I (VH genes). Again, after electrophoresis through a 1.4% agarose gel, size comparison of the fragment released due to enzyme digestion served to identify potentially positive clones.

25 The above procedures were used to confirm the presence of a potentially correct insert. However, they were not specific enough to detect small errors in the sequence (insertions, deletions and substitutions). To determine which clones contained DNA encoding complete Ig genes, each potentially positive heavy chain clone was cotransfected into COS cells with each potentially positive light chain

clone. Culture supernatants were screened by ELISA for the presence of human IgG, and then for the presence of IgG binding to α 2-antiplasmin (see below).

DNA for COS transfections was derived from midi DNA preparations described above. COS transfections were performed in 60 mm dishes. Complete
5 details of the DEAE - dextran technique employed have been described (Linsley P.S. *et al*, *J. Exp. Med.* 173: 721-730 (1991)). Typically, 1.5 μ g - 6 μ g of whole antibody is derived from small scale COS transfections

As a final confirmation, the V region inserts from the above clones were sequenced by the dideoxy nucleotide procedure.

10 **B. Production of Humanized and Chimeric Antibodies**

Once heavy and light chain vectors encoding each of the desired antibodies were qualified, sufficient quantities of chimeric and humanized antibody for testing in functional assays were needed. This was first done as a scale-up of the COS transfections using the selected vectors. Finally, stable cell lines were prepared by
15 high copy number electroporation. The electroporation protocol of Barsoum (Barsoum, *DNA and Cell Biology* 9:293-300 (1990)) was followed with the exception that 100 μ g each of the heavy and light chain vector were used (following restriction with BssHII) and the electroporation was performed in PFCHO media (PX-CELL PFCHO media, JRH Biosciences, Lenexa, Kansas).

20 Transfected cells were selected in media containing either 20 nM or 100 nM methotrexate (MTX). Culture supernatants were assayed for the presence of whole antibody using the non-specific IgG ELISA. Cells from master wells containing the most antibody in the supernatant were expanded into larger volumes. In some cases, the methotrexate concentration was also increased in order to amplify the
25 vector in the cell lines. The vector pairs in Table 4 were electroporated into DG44 CHO cells.

Table 4. Vector pairs for production of antibody		
Product	Heavy Chain Vector	Light Chain Vector
c77A3 (chimeric 77A3)	pD20-cR1.H1	pD16-cR1.L1
h77A3-1 (humanized 77A3)	pD20-hR1.H1	pD16-hR1.L1
h77A3-2 (humanized 77A3)	pD20-hR2.H1	pD16-hR1.L1
h77A3-3 (humanized 77A3)	pD20-hR3.H1	pD16-hR1.L1

C. Purification of Humanized and Chimeric Antibodies

The purification of the antibody was first performed using protein-A affinity chromatography. A Pharmacia column, sized so that 5 mg of antibody to be loaded per 1 ml of resin, was packed with Perseptive Biosystems Poros 50 A protein-A resin. The column was then sanitized according to the methods recommended by the resin supplier. The column was equilibrated with pyrogen free 10 mM sodium phosphate, 150 mM sodium chloride pH 7.0 (PBS). The cell culture supernatant was adjusted to pH of 7.0-7.5 and loaded on the column at a flow rate equal to 2 - 3 column volume/min (CV/min). The column was then washed with 15 CV pyrogen free PBS or until a stable base line has been achieved. The antibody was eluted with 20 mM glycine/HCl pH 3.0 elution buffer. The eluted peak was collected in a pyrogen free vessel that contained 1/20 CV of 1 M Tris base solution. The pH of the eluted antibody solution was adjusted to pH 8.0 with 1M Tris base immediately. The column was then cleaned with 5 CV 12 mM HCl solution. The column was stored in 20% ethanol/water at 4.0°C.

The antibody was next purified using anion exchange chromatography. A Pharmacia column, sized so that 5 - 10 mg of antibody to be loaded per 1 ml of resin, was packed with Perseptive Biosystems Poros HQ 50 anion exchange resin. The column was then sanitized according to the methods recommended by the resin

supplier. The column was equilibrated with pyrogen free 50 mM Tris/HCl, 50 mM NaCl, pH 8.0. The protein-A purified antibody adjusted to pH of 8.0 was loaded on the column with flow rate equal to 1 CV/min. The column was then washed with 5 CV pyrogen free 50 mM Tris/HCl, 1M NaCl pH 8.0. The antibody does not bind to this column under the running conditions and was present in the flowthrough fraction. The column was stored in 20% ethanol/water at 4.0°C. The antibody was then concentrated and diafiltered against PBS using a 30K cut off membrane.

D. Non-specific IgG ELISA to detect presence of antibody

This ELISA detects whole antibody (containing both heavy and light chain) and relies on a capture antibody specific for human IgG Fc region and a conjugate specific for human kappa chains. In this assay, Immunlon II flat bottom plates (Dynatech) were coated with goat anti-human IgG (Fc specific, adsorbed on mouse IgG) (Caltag, Inc. catalog #H10000) at 0.5 µg/ml in carb/bicarb buffer pH 9.6 and then blocked with PTB (PBS containing 0.05% Tween 20 and 1.0% BSA). Sample was added (either undiluted or diluted in PTB or Genetic Systems specimen diluent), the plates were incubated o/n at 4°C or for a few hours at room temperature. After washing, conjugate (goat anti-human kappa conjugated with horseradish peroxidase from Southern Biotech) was added at 1:10000 in PTB. After approximately 1 hour incubation at room temperature, plates were washed and 100 µl chromagen/substrate was added (Genetic Systems chromagen diluted 1:100 into Genetic Systems substrate). After sufficient color development (usually 5 to 15 minutes) 100 µl 1 N H₂SO₄ was added to stop the reaction. Optical densities were determined using a Biotek plate reader set at 450 and 630 nm wavelengths.

In the occasional case that none of the samples from small COS transfections showed the presence of whole antibody, similar ELISAs were performed to determine whether any light chain was being secreted. In this case,

the plates were coated with a goat anti-human kappa chain at 1 μ g/ml. The rest of the assay was done exactly as above.

5 The assay was used for three purposes. First, to screen small COS transfections that were set up to qualify various heavy and light chain vectors. In this case, the presence or absence of a signal was sufficient and it was not necessary to quantify the amount of antibody present. Second, to determine which of many master wells from CHO transfections were producing the most antibody. In this case, culture supernatants were diluted so that relative signals could be compared and the master wells containing the most antibody could be distinguished and thus
10 selected for cloning and expansion. Thirdly, to determine amounts of antibody, either in culture supernatants or following purification. In this case, a standard consisting of either a chimeric or human IgG1 or a human myeloma IgG2 were used. Both standard and sample were serially diluted (2x) across a plate and sample concentration relative to standard was determined by comparing position of the
15 curves. The concentrations thus determined were used for following antibody production during the cloning and amplification process and for determining specific activity in the antigen binding ELISA and any of the functional assays.

E. ELISAs to show that antibody is capable of binding to antigen

20 This ELISA relies on an antigen capture and a human kappa chain specific conjugate. It was used for two purposes. Initially, to qualify a vector, supernatants from COS transfections were screened for the ability of antibody to bind to antigen. Vectors passing this test were then submitted to DNA sequencing. Secondly, to determine relative antigen binding ability of the various chimeric and humanized antibodies. This ELISA is very similar to the non-specific IgG ELISA described
25 above except that the plates were coated with α 2-antiplasmin (obtained from American Diagnostica) at 1 μ g/ml in PBS.

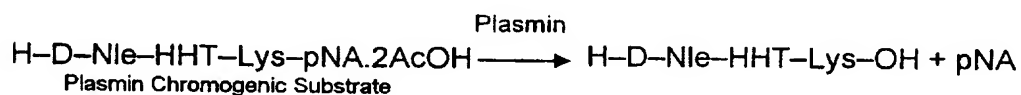
To determine relative antigen binding ability of various antibodies, scatter plots were used with log antibody concentration along the X axis and optical density

along the Y axis. Antigen concentration was determined either from the non-specific ELISA or based on optical density of purified preparations. All three forms of humanized antibody (h77A3-1, -2, and -3) show antigen binding similar to that of the chimeric antibody. Comparisons were not made with the murine antibody (m77A3) because the m77A3 cannot be detected in the assay as described (the antibody-conjugate used in the second step recognizes only human constant regions).

F. Functional Assays

Two functional assays were performed. The first, known as the "plasmin assay with chromogenic substrate" is based on the ability of plasmin to convert Spectrozyme PL, H-D-Nle-HHT-Lys-pNA.2AcOH into pNA, which absorbs light at 405 nm. If unblocked α 2-antiplasmin is present, little or no conversion occurs. Active antibody is capable of blocking the inhibitory activity of α 2-antiplasmin. The second assay, the clot lysis assay, is a measure of the ability of antibody along with urokinase to lyse preformed clots.

The plasmin assay with chromogenic substrate is designed based on the action of plasmin on its chromogenic substrate according to the reaction:



The generation of pNA was monitored by the increase in absorption at 405 nm using a SpectraMax 250 spectrophotometer. The addition of α 2-antiplasmin inhibits the plasmin activity and no increase in absorption at 405 nm will be observed. Premixing of α 2-antiplasmin with functional antibody blocks the ability of α 2-antiplasmin to inhibit the plasmin activity. Plasmin activity was measured as the initial rate of color development.

Assays are performed in 96 well microtiter plates. The chromogenic substrate Spectrozyme PL, H-D-Nle-HHT-Lys-pNA.2AcOH, human plasmin, and human α 2-antiplasmin were purchased from American Diagnostica. Stock and

working solutions are prepared as follows: Spectrozyme PL stock solution - 10 mM in H₂O; Spectrozyme PL working solution - 1:12.5 dilution of stock solution in H₂O; human plasmin stock solution - 0.2 mg/ml in 50% glycerol, 50% 2 mM HCl; human plasmin working solution - 1 : 12.5 dilution of stock solution in 0.11 mM HCl, which must be prepared immediately before use; human α 2-antiplasmin stock solution - 0.2 mg/ml in PBS; and human α 2-antiplasmin working solution - 1:15 dilution of stock solution in PBS. Stock solutions were stored at -70 and should not be refrozen after thawing.

Reagents are added in the following order, with mixing after each addition: 80 ul antibody or PBS, 40 ul α 2-antiplasmin working solution, 40 ul plasmin working solution, and 40 ul Spectrazyme PL working solution. R is the rate of color development. R_p, which represents maximum plasmin activity, is determined in wells lacking both antibody and α 2-antiplasmin. R_o, which represents minimal plasmin activity, is determined in wells lacking antibody. R_s is the rate of color development in the sample. Antibody activity is calculated as $(R_s - R_o)/(R_p - R_o) * 100$. Values should range between 0% and 100%. Antibody activity was plotted vs. amount antibody (on a log scale). Curves generated by test antibody and standard (usually murine 77A3) were compared.

The data for murine 77A3, c77A3, and h77A3-1 are shown in Figure 20. The curves for murine and chimeric 77A3 were superimposable. The curve for h77A3-2 indicates a potential small loss in activity (20-30%).

The clot lysis assays were performed as follows. Test clots were formed in 96-well Corning #25805 microtiter plates by mixing 25 uL 16 mM CaCl₂, 50 uL of pooled human plasma, and 25 uL of 4 NIH unit/ml of human alpha-thrombin (Sigma) in 30mM Hepes buffer, pH 7.40. Plates were incubated overnight at room temperature to allow clots to achieve maximum clot turbidities. Clot lysis was initiated by adding 10 uL of antibody to give 5 or 10 ug/well and 100 uL of urokinase to give 1, 3 or 5 units of urokinase/well (Abbott Labs) at pH 7.40. Plates were mixed on a table top microplate vortexer for 30 sec before the initial reading at 405 nm to get values corresponding to 0% lysis. Plates were sealed with Corning

sealing tape #430454 and incubated at 37°C. During the course of 24 hrs, the decrease of turbidity was measured at 405 nm to quantify the progress of clot lysis.

The results of a clot lysis experiment of humanized 77A3-1 indicate that h77A3-1 enhances clot lysis dramatically in comparison to buffer controls in each of the conditions tested. There was significant separation between the humanized and murine 77A3 in clots containing 5 ug antibody in the presence of 1 or 3 units of urokinase indicating that humanized 77A3 was somewhat less active than murine 77A3, even though the lysis profiles were similar at the remaining four conditions tested. It should be noted that murine RWR, a monoclonal antibody with a 10-fold lower affinity than murine 77A3, causes no lysis at 10 ug per clot in the presence of 1 unit of urokinase and would give a lysis profile like buffer control.

Example 5

Preparation and Characterization of Single Chain Fv Fragments

A. Design and expression of sFv form of 77A3

The sFv fragment of an antibody is most commonly obtained by the tandem expression of the variable region of the antibody heavy chain along with the variable region of the antibody light chain spaced by a linker of 15-20 amino acids. sFv fragments are expected to have superior clot penetration to parent antibodies. Two constructs, p53-6 and p52-12, were prepared using murine variable regions with a VH-(linker)-VL polarity using YPRSIYIRRRHPSPSLTT (SEQ ID NO:73) as linker 1 for sFv77A3-1 and GGSGSGGSGSGGSGS (SEQ ID NO:74) as linker 2 for sFv77A3-2. Both constructs were cloned into the pET-22b vector from Novagen and transformed into the BL21 (DE3) strain of *E. coli* grown in minimal M9 media. Though the majority of the His-tagged product was found in inclusion bodies, supernatants of cell lysate contained sufficient quantities of soluble sFv fragments for nickel-column purification.

-73-

sFv77A3-2 present in fractions 7-11 collected from a nickel-column gave a single Coomassie staining band with a MW about 30,000 agreeing well with the calculated MW of 29,986. A similar but more weakly staining gel was obtained for sFv77A3-1.

5

B. Activity of sFv77A3-1 and sFv77A3-2

10

Preparations of both sFv77A3-1 and sFv77A3-2 were tested for alpha2-antiplasmin binding activity in a competition binding assay. Microplate wells coated with 77A3 were treated with mixtures of biotinylated-human alpha2-antiplasmin and either sFv77A3-1 or sFv77A3-2 along with positive control 77A3 and negative control, mAb-59D8. Increasing quantities of 77A3 prevented binding of biotinylated-human alpha2-antiplasmin whereas negative control 59D8 had little effect as a competitive inhibitor. With concentrations of test samples estimated by intensity of Coomassie stained bands, both sFv77A3-1 and sFv77A3-2 completely inhibited the binding of biotinylated-human α 2-antiplasmin with a profile of inhibition nearly superimposable to the parental 77A3 reference.

15

20

Idiotypic markers present on 77A3 were probed with a sandwich ELISA using a biotinylated polyclonal reagent rendered specific by multiple immunoadsorption steps through columns bearing immunoglobulins from man, mouse, baboon and cynomologous monkey (P Stenzel-Johnson & D Yelton, Seattle). Microplate wells were coated with 77A3 and 59D8 as controls along with sFv77A3-1 and sFv77A3-2. It is evident that 77A3 control and both sFv fragments bear idiotypic markers at each dose tested indicating that the sFv fragments "look" like the parental 77A3.

25

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

5 The disclosure of all references, patent application, and patents referred to herein are hereby incorporated by reference.

Applicant's or agent's file reference number	0609.432PC01	International application No. TBA
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(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>7</u> , line <u>12</u>	
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Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 20 September 1996	Accession Number HB-12192
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
Hybridoma cell line, 77a3	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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Date of deposit 19 September 1997	Accession Number TBA
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77A3-HC	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 19 September 1997	Accession Number TBA
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77A3-IC	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>18</u> , line <u>22</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 19 September 1997	Accession Number TBA
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
49C9-HC	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit") Accession number will be furnished at a later date.	

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Applicant's or agent's file reference number	09.432PC01	International application TBA
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>18</u> , line <u>19</u>	
B. IDENTIFICATION OF DEPOSIT	
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Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 19 September 1997	Accession Number TBA
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
49C9-IC	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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Authorized officer <i>Spiegel Menden</i> (763) 533745

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Applicant's or agent's file reference number	09.432PC01	International application TBA
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>18</u> , line <u>23</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 19 September 1997	Accession Number TBA
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
70B11-HC	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit") Accession number will be furnished at a later date.	

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Authorized officer	<i>Sybil Menden</i> <i>(7.5) 25-3745</i>

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Authorized officer	

Applicant's or agent's file reference number	0609.432PC01	International application no	TBA
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>18</u> , line <u>20</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 19 September 1997	Accession Number TBA
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
70B11-LC	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit") Accession number will be furnished at a later date.	
<input checked="" type="checkbox"/> For receiving Office use only This sheet was received with the international application	<input type="checkbox"/> For International Bureau use only This sheet was received by the International Bureau on:
Authorized officer <i>[Signature]</i> (703) 205-3745	Authorized officer

What Is Claimed Is:

1. An immunologic molecule wherein said immunologic molecule is capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins.

2. The immunologic molecule of claim 1, wherein said immunologic molecule is a chimeric antibody.

3. The immunologic molecule of claim 1, wherein said immunologic molecule is a humanized antibody.

4. The immunologic molecule of claim 1, wherein said immunologic molecule is an antibody fragment.

5. The immunologic molecule of claim 1, wherein said immunologic molecule is a monoclonal antibody.

6. The immunologic molecule of claim 1, wherein said immunologic molecule comprises amino acids 1 to 107 of SEQ ID NO:9 and amino acids 1 to 119 of SEQ ID NO:15.

7. The immunologic molecule of claim 1, wherein said immunologic molecule comprises amino acids 1 to 107 of SEQ ID NO:5 and amino acids 1 to 119 of SEQ ID NO:11.

8. The immunologic molecule of claim 1, wherein said immunologic molecule comprises amino acids 1 to 107 of SEQ ID NO:7 and amino acids 1 to 119 of SEQ ID NO:13.

9. The immunologic molecule of claim 1, selected from the group consisting of:

5 (a) an immunologic molecule, wherein the CDR1 region of the light chain of said immunologic molecule comprises amino acids 26 to 32 of SEQ ID NO:75;

(b) an immunologic molecule, wherein the CDR2 region of the light chain of said immunologic molecule comprises amino acids 50 to 52 of SEQ ID NO:75;

10 (c) an immunologic molecule, wherein the CDR3 region of the light chain of said immunologic molecule comprises amino acids 91 to 96 of SEQ ID NO:75;

(d) an immunologic molecule, wherein the CDR1 region of the heavy chain of said immunologic molecule comprises amino acids 26 to 32 of SEQ ID NO:79;

15 (e) an immunologic molecule, wherein the CDR2 region of the heavy chain of said immunologic molecule comprises amino acids 53 to 56 of SEQ ID NO:79; and

20 (f) an immunologic molecule, wherein the CDR3 region of the heavy chain of said immunologic molecule comprises amino acids 100 to 107 of SEQ ID NO:79.

10. The immunologic molecule of claim 1, selected from the group consisting of:

25 (a) an immunologic molecule, wherein the CDR1 region of the light chain of said immunologic molecule comprises amino acids 26 to 32 of SEQ ID NO: 76;

(b) an immunologic molecule, wherein the CDR2 region of the light chain of said immunologic molecule comprises amino acids 50 to 52 of SEQ ID NO:76;

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(c) an immunologic molecule, wherein the CDR3 region of the light chain of said immunologic molecule comprises amino acids 91 to 96 of SEQ ID NO:76;

5 (d) an immunologic molecule, wherein the CDR1 region of the heavy chain of said immunologic molecule comprises amino acids 26 to 32 of SEQ ID NO:80;

(e) an immunologic molecule, wherein the CDR2 region of the heavy chain of said immunologic molecule comprises amino acids 53 to 56 of SEQ ID NO:80; and

10 (f) an immunologic molecule, wherein the CDR3 region of the heavy chain of said immunologic molecule comprises amino acids 100 to 107 of SEQ ID NO:80.

15 11. The monoclonal antibody of claim 5, wherein said monoclonal antibody is 77A3.

12. The monoclonal antibody of claim 5, wherein said monoclonal antibody is 49C9.

20 13. The monoclonal antibody of claim 5, wherein said monoclonal antibody is 70B11.

14. A method of making the monoclonal antibody of claim 5 comprising:

25 (a) immunizing an animal with α 2-antiplasmin or fragment thereof;

(b) fusing cells from the animal with tumor cells to make a hybridoma cell line;

(c) cloning the hybridoma cell line;

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(d) selecting for the monoclonal antibody capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins; and

(e) obtaining the monoclonal antibody.

5

15. A hybridoma cell line which produces the monoclonal antibody of claim 5.

10

16. The hybridoma cell line of claim 15, wherein said hybridoma cell line is ATCC Accession No. HB-12192.

17. A method of making the hybridoma cell line of claim 15 comprising:

15

(a) immunizing an animal with α 2-antiplasmin or fragment thereof;

(b) fusing the cells from the animal with tumor cells to make the hybridoma cell line; and

20

(c) obtaining the hybridoma cell line which produces the monoclonal antibody capable of binding to both (1) human and nonhuman circulating α 2-antiplasmins and (2) human and nonhuman fibrin crosslinked α 2-antiplasmins.

25

18. A nucleic acid molecule, selected from the group consisting of:

(a) a nucleic acid molecule comprising a nucleotide sequence encoding for amino acids 1 to 107 of SEQ ID NO:5;

(b) a nucleic acid molecule comprising a nucleotide sequence encoding for amino acids 1 to 107 of SEQ ID NO:7;

(c) a nucleic acid molecule comprising a nucleotide sequence encoding for amino acids 1 to 107 of SEQ ID NO:9;

30

(d) a nucleic acid molecule comprising a nucleotide sequence encoding for amino acids 1 to 107 of SEQ ID NO:75;

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(e) a nucleic acid molecule comprising a nucleotide sequence encoding for amino acids 1 to 119 of SEQ ID NO:11;

(f) a nucleic acid molecule comprising a nucleotide sequence encoding for amino acids 1 to 119 of SEQ ID NO:13;

5 (g) a nucleic acid molecule comprising a nucleotide sequence encoding for amino acids 1 to 119 of SEQ ID NO:15; and

(h) a nucleic acid molecule comprising a nucleotide sequence encoding for amino acids 1 to 119 of SEQ ID NO:79.

10 19. A method for treating pulmonary embolism, myocardial infarction, or thrombosis in a patient comprising administering a therapeutically effective amount of an immunologic molecule of claim 1 to said patient.

15 20. The method of claim 19, wherein said immunologic molecule is a monoclonal antibody.

21. The method of claim 20, wherein said monoclonal antibody is 77A3.

20 22. The method of claim 19, wherein said immunologic molecule is administered by continuous intravenous infusion or by bolus.

25 23. A method of treatment for pulmonary embolism, myocardial infarction, or thrombosis in a patient which comprises co-administering to a patient in need of such treatment:

(a) a therapeutically effective amount of an immunologic molecule of claim 1; and

30 (b) a therapeutically effective amount of a thrombolytic agent, wherein said immunologic molecule (a) is different from said thrombolytic agent (b), thereby treating said patient.

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24. The method of claim 23, wherein said immunologic molecule is a monoclonal antibody.

25. The method of claim 24, wherein said monoclonal antibody is 77A3.

26. The method of claim 23, wherein said thrombolytic agent is plasmin.

27. The method of claim 23, wherein said thrombolytic agent is an anti-coagulant which inhibits fibrin.

28. The method of claim 27, wherein said anti-coagulant is selected from the group consisting of heparin, hirudin and activated protein C.

29. The method of claim 23, wherein said thrombolytic agent is an anti-coagulant which inhibits platelets.

30. The method of claim 23, wherein said thrombolytic agent is a plasminogen activator.

31. The method of claim 30, wherein said plasminogen activator is selected from the group consisting of streptokinase, prourokinase, urokinase, tissue-type plasminogen activator, staphylokinase, and vampire bat plasminogen activator.

32. The method of claim 23, wherein both said immunologic molecule (a) and said thrombolytic agent (b) are provided to said patient by an intravenous infusion or by an intravenously injected bolus.

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33. The method of claim 23, wherein said patient is provided with a first bolus containing said immunologic molecule (a) and a subsequently administered second bolus containing said thrombolytic agent (b).

5 34. The method of claim 23, wherein:

(1) said immunologic molecule (a) is provided to said patient at a dose of between 3 to 600 nmole per kg of patient weight; and

(2) said thrombolytic agent (b) is provided to said patient at a dose of between 0.01 to 3.0 mg per kg of patient weight.

10 35. A kit useful for carrying out the method of claim 23, being compartmentalized in close confinement to receive two or more container means therein, which comprises:

15 (1) a first container containing a therapeutically effective amount of said immunologic molecule (a); and

(2) a second container containing a therapeutically effective amount of said thrombolytic agent (b), wherein said immunologic molecule (a) is different from said thrombolytic agent (b).

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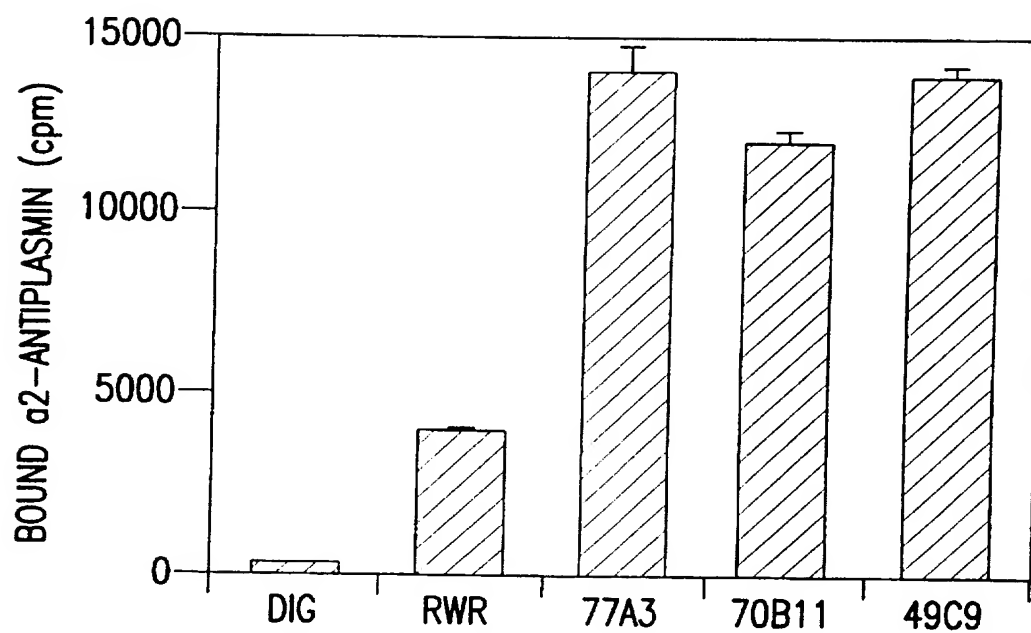


FIG.1

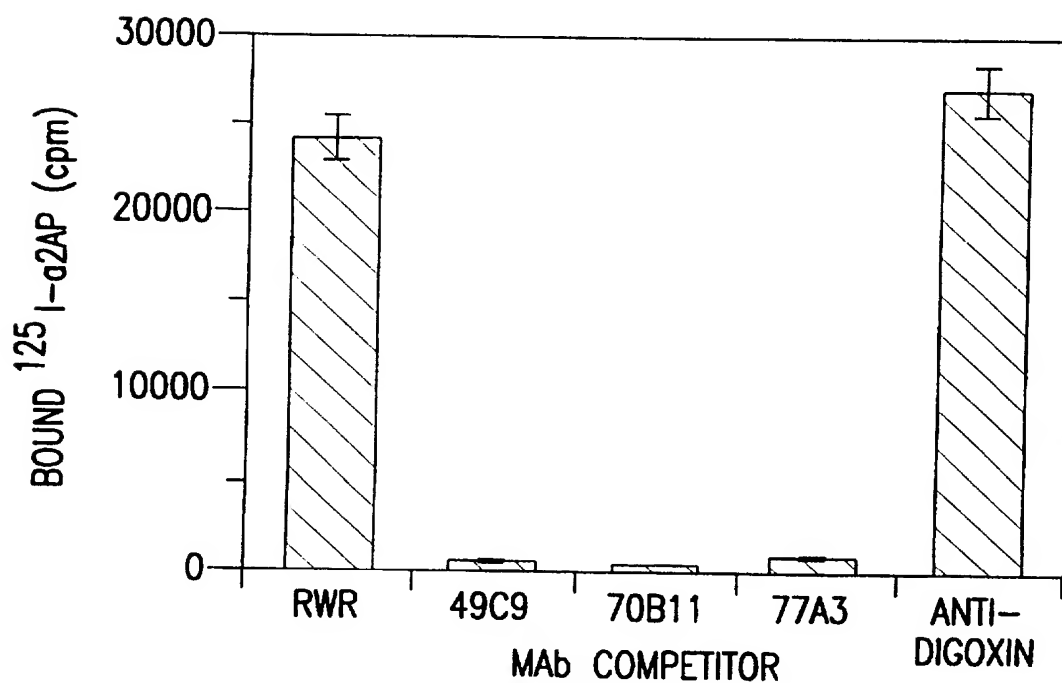


FIG.2

SUBSTITUTE SHEET (RULE 26)

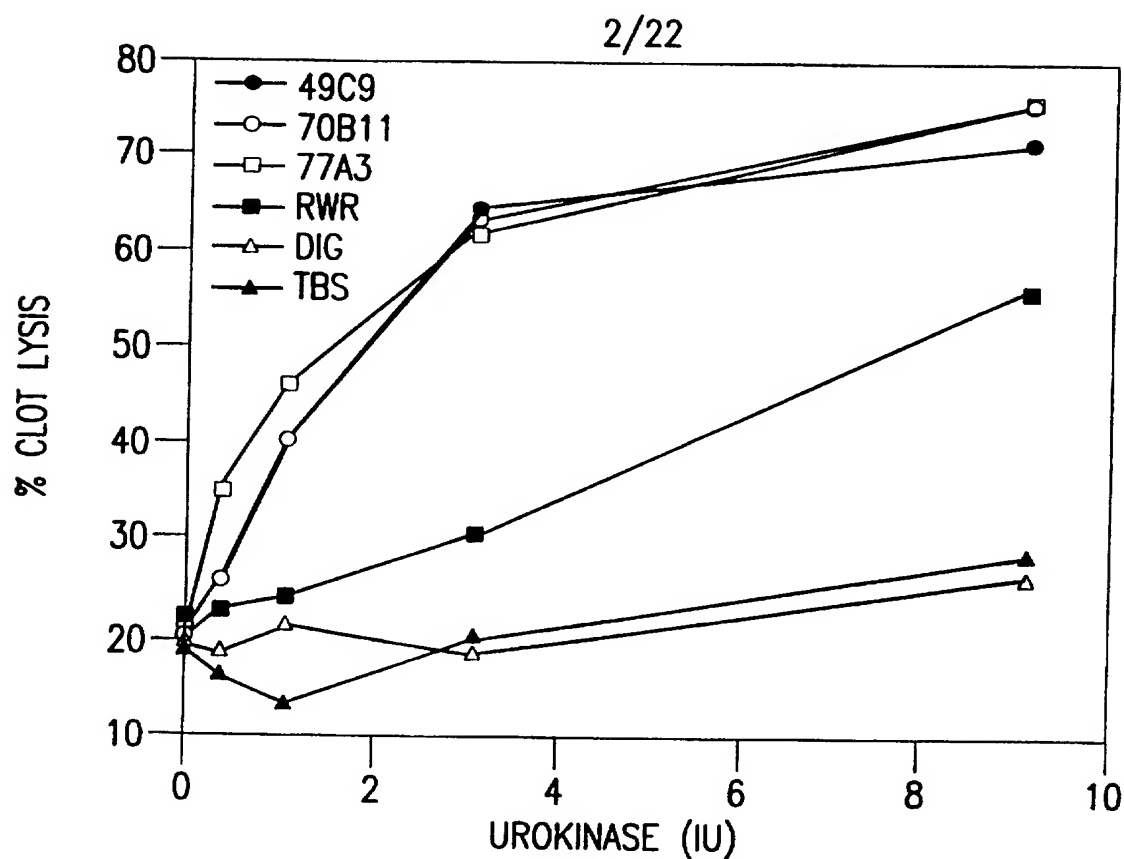


FIG.3

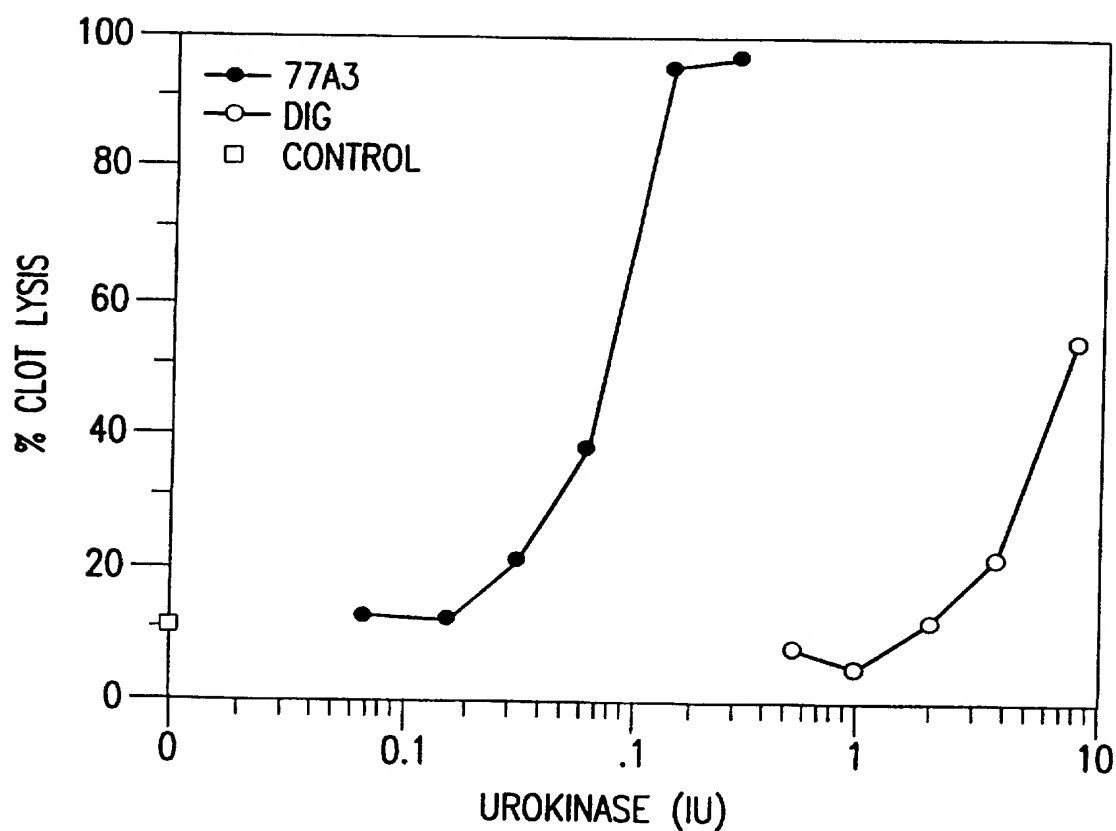


FIG.4

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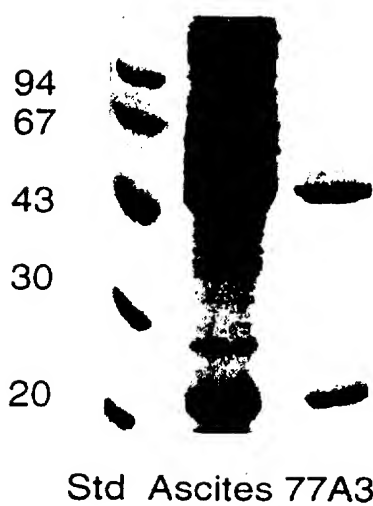


FIG.5

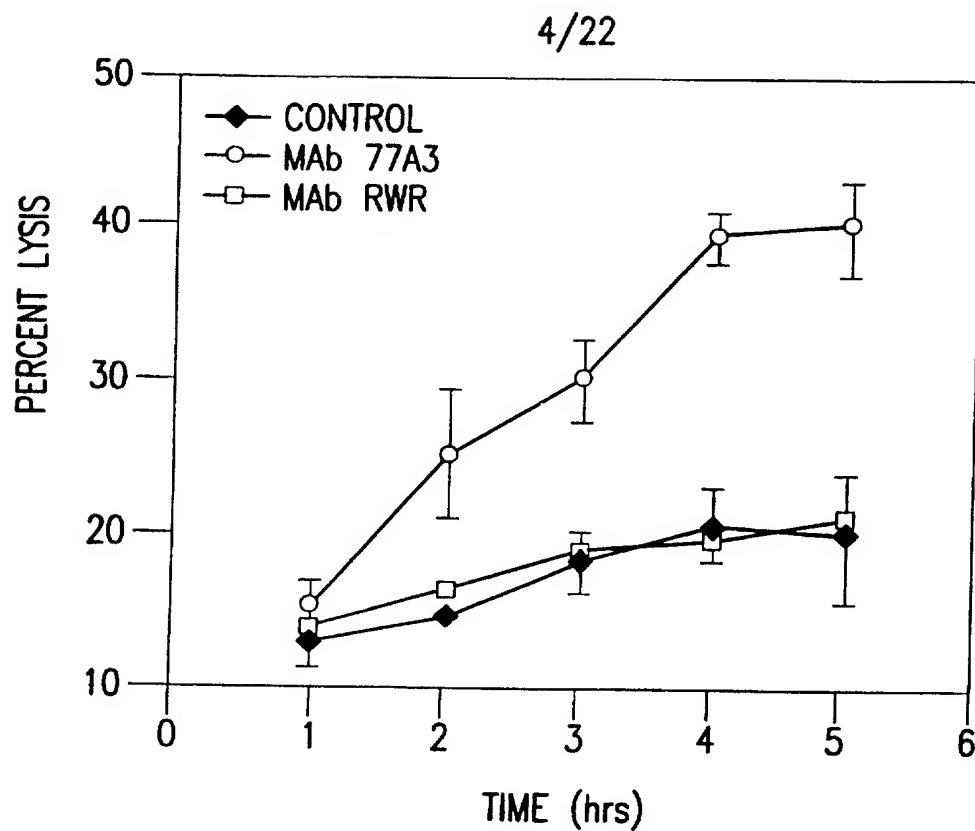


FIG.6

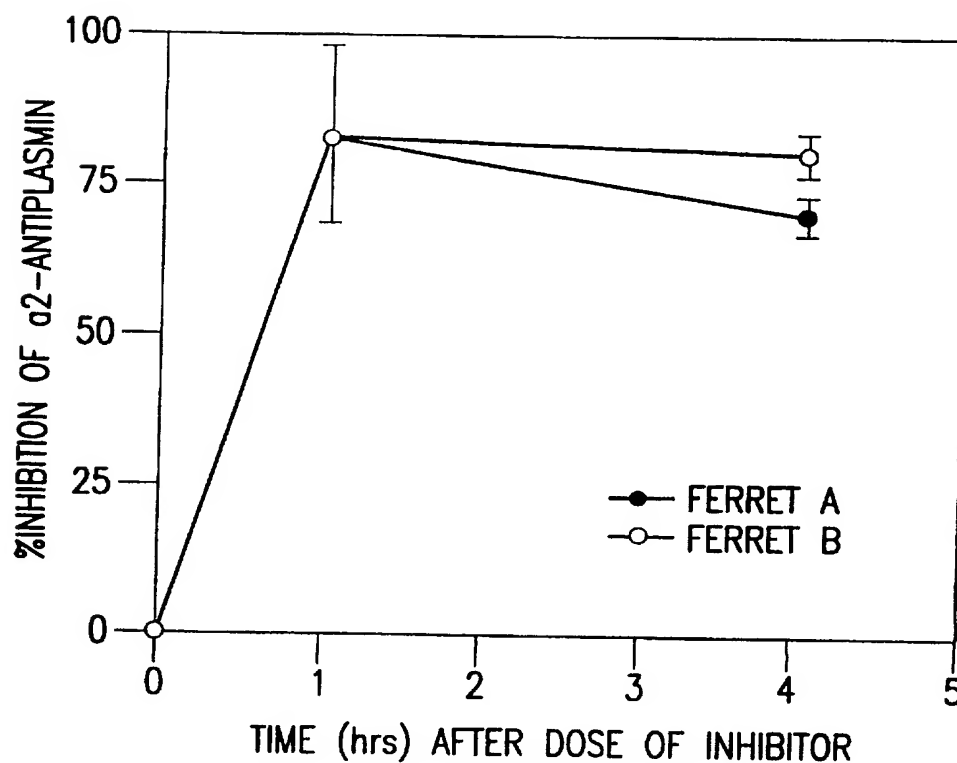


FIG.7

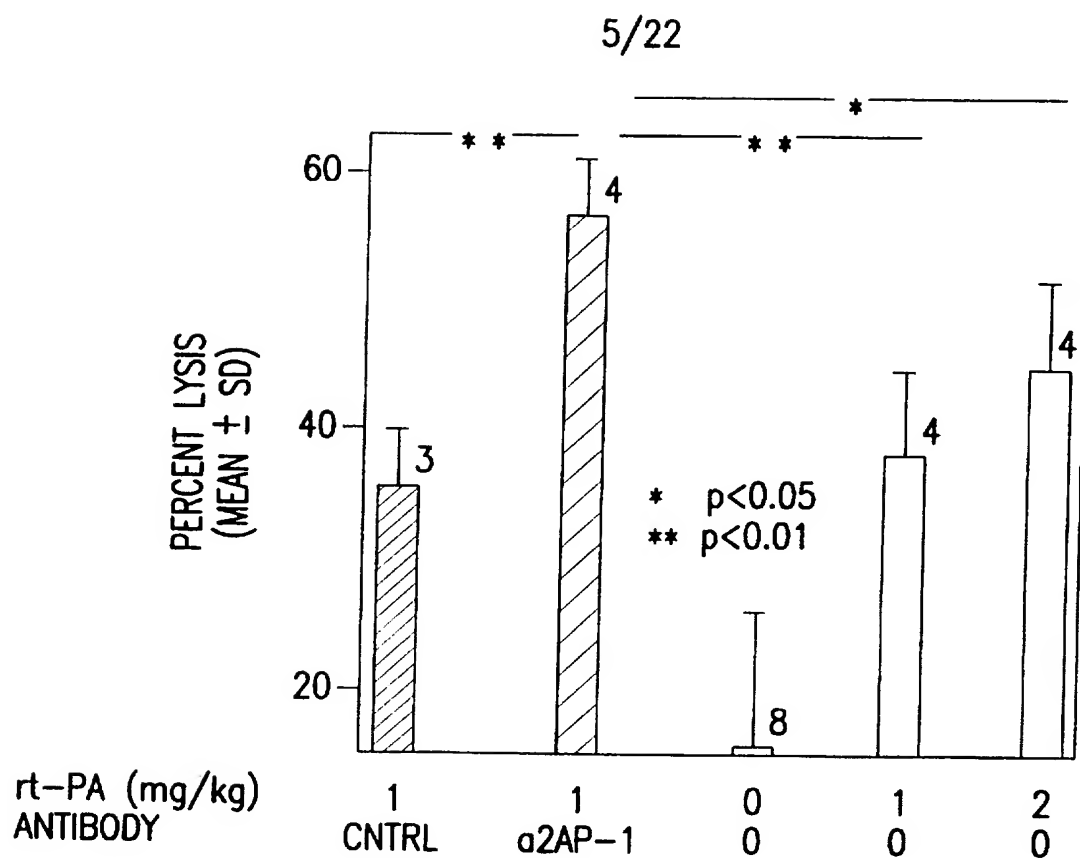


FIG.8

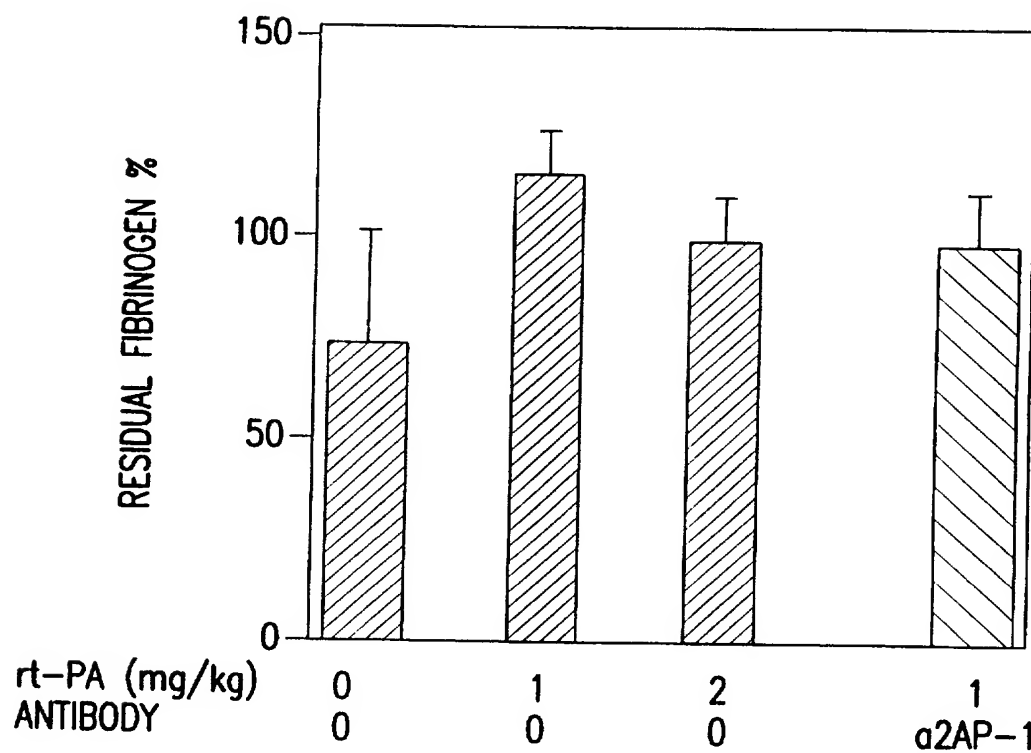


FIG.9

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MAb Light Chain	Amino Terminal Sequence
49C9	xIQMTQSPASLSASV
70B11	DIQMT
77A3	xIQMTQSPASLSASV

FIG.10

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```

      10      20      30      40      50      60
      *      *      G      *      *      *
ATGAGTGTGC TCACTCAGGT CCTGGCGTTG CTGCTGCTGT GGCTTACAGG TGCCAGATGT
M S V L T Q V L G L L L L W L T G A R C>
      A

      70      80      90      100      110      120
      *      *      *      *      *      *
GACATCCAGA TGA CTCAGTC TCCAGCCTCC CTATCTGCAT CTGTGGGAGA AACTGTCACC
D I Q M T Q S P A S L S A S V G E T V T>

      130      140      150      160      170      180
      *      *      *      *      *      *
ATCACATGTC GAGCAAGTGG GAATATTCAC AATTATTTAG CATGGTATCA GCAGAAACAG
I T C R A S G N I H N Y L A W Y Q Q K Q>

      190      200      210      220      230      240
      *      *      *      *      *      *
GGAAAATCTC CTCAGCTCCT GGTCTATAAT GCAAAAACCT TAGCAGATGG TGTGCCATCA
G K S P Q L L V Y N A K T L A D G V P S>

      250      260      270      280      290      300
      *      *      *      *      *      *
AGGTT CAGTG GCAGTGGATC AGGAACACAA TTTTCTCTCA GGATCAACAG CCTGCAGCCT
R F S G S G S G T Q F S L R I N S L Q P>

      310      320      330      340      350      360
      *      *      *      *      *      *
GAAGATTTTG GGAGTCATTA CTGTCAACAT TTTTGGACCA CTCCGTGGAC GTTCGGTGGA
E D F G S H Y C Q H F W T T P W T F G G>

      370      380
      *      *
GGCACCAAGC TGGAAATCAA A
G T K L E I K

```

FIG. 11

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10 20 30 40 50 60
* * * * *
ATGAGTGTGC TCACTCAGGT CCTGGGGTTG CTGCTGCTGT GGCTTACAGG TGCCAGATGT
M S V L T Q V L G L L L L W L T G A R C>

70 80 90 100 110 120
* * * * *
GACATCCAGA TGA CTCAGTC TCCAGCCTCC CTATCTGCAT CTGTGGGAGA AACTGTCACC
D I Q M T Q S P A S L S A S V G E T V T>

130 140 150 160 170 180
* * * * *
GTCACATGTC GAGCAAGTGG GAATATTCAC AATTATTTAG CATGGTATCA GCAGAAACAG
V T C R A S G N I H N Y L A W Y Q Q K Q>

190 200 210 220 230 240
* * * * *
GGAAAATCTC CTCAGCTCCT GGTCTATAAT GCAAGAACCT TAGCAGATGG TGTGCCATCA
G K S P Q L L V Y N A R T L A D G V P S>

250 260 270 280 290 300
* * * * *
AGGTTTCAGTG GCAGTGGATC AGGAACACAA TATTCTCTCA AGATCAACAG CCTGCAGCCT
R F S G S G S G T Q Y S L K I N S L Q P>

310 320 330 340 350 360
* * * * *
GAAGATTTTG GGAGTTATTA CTGTCAACAT TTTTGGAGTA ATCCGTGGAC GTTCGGTGGA
E D F G S Y Y C Q H F W S N P W T F G G>

370 380
* *
GGCACCAAGC TGGAAATCAA
G T K L E I K

FIG.12

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10	20	30	40	50	60
*	*	*	*	*	*
ATGAGTGTGC TCACTCAGGT CCTGGCGTTG CTGCTGCTGT GGCTTACAGG TGCCAGATGT					
M S V L T Q V L A L L L L W L T G A R C>					
70	80	90	100	110	120
*	*	*	*	*	*
GACATCCAGA TGA CTCAGTC TCCAGCCTCC CTATCTGCAT CTGTGGGAGA AACTGTCACC					
D I Q M T Q S P A S L S A S V G E T V T>					
130	140	150	160	170	180
*	*	*	*	*	*
ATCACATGTC GAGCAAGTGG GAATATTCAC AATTATTTAG CATGGTATCA GCAGAAACAG					
I T C R A S G N I H N Y L A W Y Q Q K Q>					
190	200	210	220	230	240
*	*	*	*	*	*
GGAAAATCTC CTCAACTCCT GGTCTATAAT GCAAAAACCT TAGCAGATGG TGTGCCATCA					
G K S P Q L L V Y N A K T L A D G V P S>					
250	260	270	280	290	300
*	*	*	*	*	*
AGGTTTCAGTG GCAGTGGATC AGGAACACAA TTTTCTCTCA AGATCAACAG CCTGCAGCCT					
R F S G S G S G T Q F S L K I N S L Q P>					
310	320	330	340	350	360
*	*	*	*	*	*
GAAGATTTTG GGAGTCATTA CTGTCAACAT TTTTGGACCA CTCCGTGGAC GTTCGGTGGA					
E D F G S H Y C Q H F W T T P W T F G G>					
370	380				
*	*				
GGCACCAAGC TGGAAATCAA A					
G T K L E I K					

FIG. 13

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H2 HC (49c9 heavy chain)

```

9  * 19  * 29  * 39  * 49  * 59  *
ATGGMTTGG GTGTGGAMCT TGCTATTCTT GATGGCAGCT GCCCAAAGTC TCCAAGCACA
M A W V W N L L F L M A A A Q S L Q A Q>
D

69  * 79  * 89  * 99  * 109  * 119  *
GATCCAGTTG GTGCAGTCTG GACCTGAGCT GAAGAAGCCT GGAGAAACAG TCAAGAICTC
I Q L V Q S G P E L K K P G E T V K I S>

129  * 139  * 149  * 159  * 169  * 179  *
CTGCAAGGCC TCTGGGTATA CCTTCACAAA CTATGGAATG AACTGGGTGA AGCAGGCTCC
C K A S G Y T F T N Y G M N W V K Q A P>

189  * 199  * 209  * 219  * 229  * 239  *
AGGAAAGGGT TTAAAGTGA TGGGCTGGAT AAACACCAAG AGTGGAGAGC CAACATATGC
G K G L K W M G W I N T K S G E P T Y A>

249  * 259  * 269  * 279  * 289  * 299  *
TGAAGAGTTC AAGGGACGGT TTGTCTTCTC TTTGGAAACC TCTGCCAGCA CTGCCCATTT
E E F K G R F V F S L E T S A S T A H L>

309  * 319  * 329  * 339  * 349  * 359  *
GCAGATCAAG AATTTCAGAA ATGAGGACAC GGCTACATAT TTCTGTGCAA GATGGGTACC
Q I K N F R N E D T A T Y F C A R W V P>

369  * 379  * 389  * 399  * 409  *
TGGGACCTAT GCTATGGACT ACTGGGGTCA AGGAACCTCA GTCACCGTCT CCTCA
G T Y A M D Y W G Q G T S V T V S S>

```

FIG.14

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H3 HC (70B11 heavy chain)

```

10 * 20 * 30 * 40 * 50 * 60 *
ATGGMTTGGG TGTGGAMCTT GCTATTCCTG ATGGCAGCTG CCCAAAGTAT CCAAGCACAG
M A W V W N L L F L M A A A Q S I Q A Q>
D

70 * 80 90 100 110 120
ATCCAGTTGG TGCAGTCTGG ACCTGAGCTG AAGAGCCTG GAGAGACAGT CAAGATCTCC
I Q L V Q S G P E L K K P G E T V K I S>

130 * 140 150 160 170 180
TGCAAGGCTT CTGGGTATAC CTTACACAAAG TATGGAATGA ACTGGGTGAA GCAGGCTCCA
C K A S G Y T F T K Y G M N W V K Q A P>

190 * 200 210 220 230 240
GGAAGGGTT TAAAGTGGAT GGGCTGGATA AACACCAACA GTGGAGAGCC AACATATGCT
G K G L K W M G W I N T N S G E P T Y A>

250 * 260 270 280 290 300
GAAGAGTTCA AGGGACGGTT TGCCTTCTCT TTGGAACCT CTGCCAGCAC TGCCTATTTG
E E F K G R F A F S L E T S A S T A Y L>

310 * 320 330 340 350 360
CAGATCAACA ACCTCAAAAA TGAGGACTCG GCTACATATT TCTGTGCAAG ATGGGTACCT
Q I N N L K N E D S A T Y F C A R W V P>

370 * 380 390 400 410
GGGACCTATG CTATGGACTA CTGGGGTCAA GGAACCTCAG TCACCGTCTC CTCA
G T Y A M D Y W G Q G T S V T V S S>

```

FIG.15

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H4 HC (77A3 heavy chain)

```

10 *      20 *      30 *      40 *      50 *      60 *
ATGGMITGGG TGTGGAMCTT GCTATTCTTG ATGGCAGCTG CCCAAAGTAT CCAAGCACAG
M A W V W N L L F L M A A A Q S I Q A Q>
D

70 *      80 *      90 *      100 *      110 *      120 *
ATCCAGTTGG TGCAGTCTGG ACCTGAGCTG AAGAGCCTG GAGAAACAGT CAAGATCTCC
I Q L V Q S G P E L K K P G E T V K I S>

130 *      140 *      150 *      160 *      170 *      180 *
TGCAAGGCTT CTGGGTATAC CTTCAACAAC TATGGAATGA ACTGGGTGAA GCAGGCTCCA
C K A S G Y T F T N Y G M N W V K Q A P>

190 *      200 *      210 *      220 *      230 *      240 *
GGAAAGGTT TAAAGTGGAT GGGCTGGATA AACACCAAGA GTGGAGAGCC AACATATGCT
G K G L K W M G W I N T K S G E P T Y A>

250 *      260 *      270 *      280 *      290 *      300 *
GAAGAGTTCA AGGACGGTT TGCCTTCTCT TTGGAACCT CTGCCAGCAC TGCCAATTTG
E F K G R F A F S L E T S A S T A N L>

310 *      320 *      330 *      340 *      350 *      360 *
CAGATCAAGA ACCTCAAAA TGAGGACACG GCTACATATT TCTGTGCAAG ATGGGTACCT
Q I K N L K N E D T A T Y F C A R W V P>

370 *      380 *      390 *      400 *      410 *
GGGACCTATG CCATGGACTA CTGGGTCAA GGAACCTCAG TCACCGTCTC CTCA
G T Y A M D Y W G Q G T S V T V S S

```

FIG.16

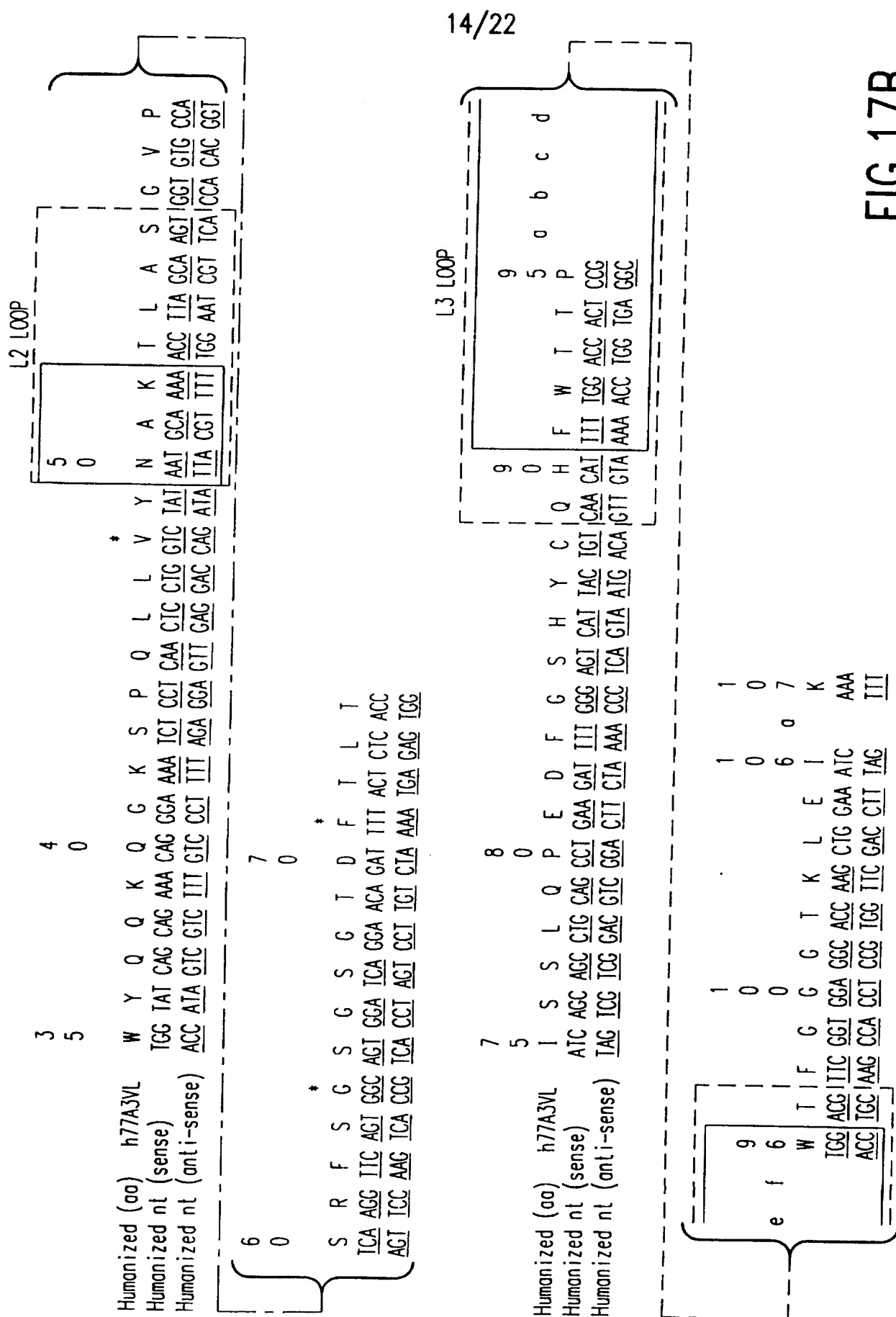


FIG. 17B

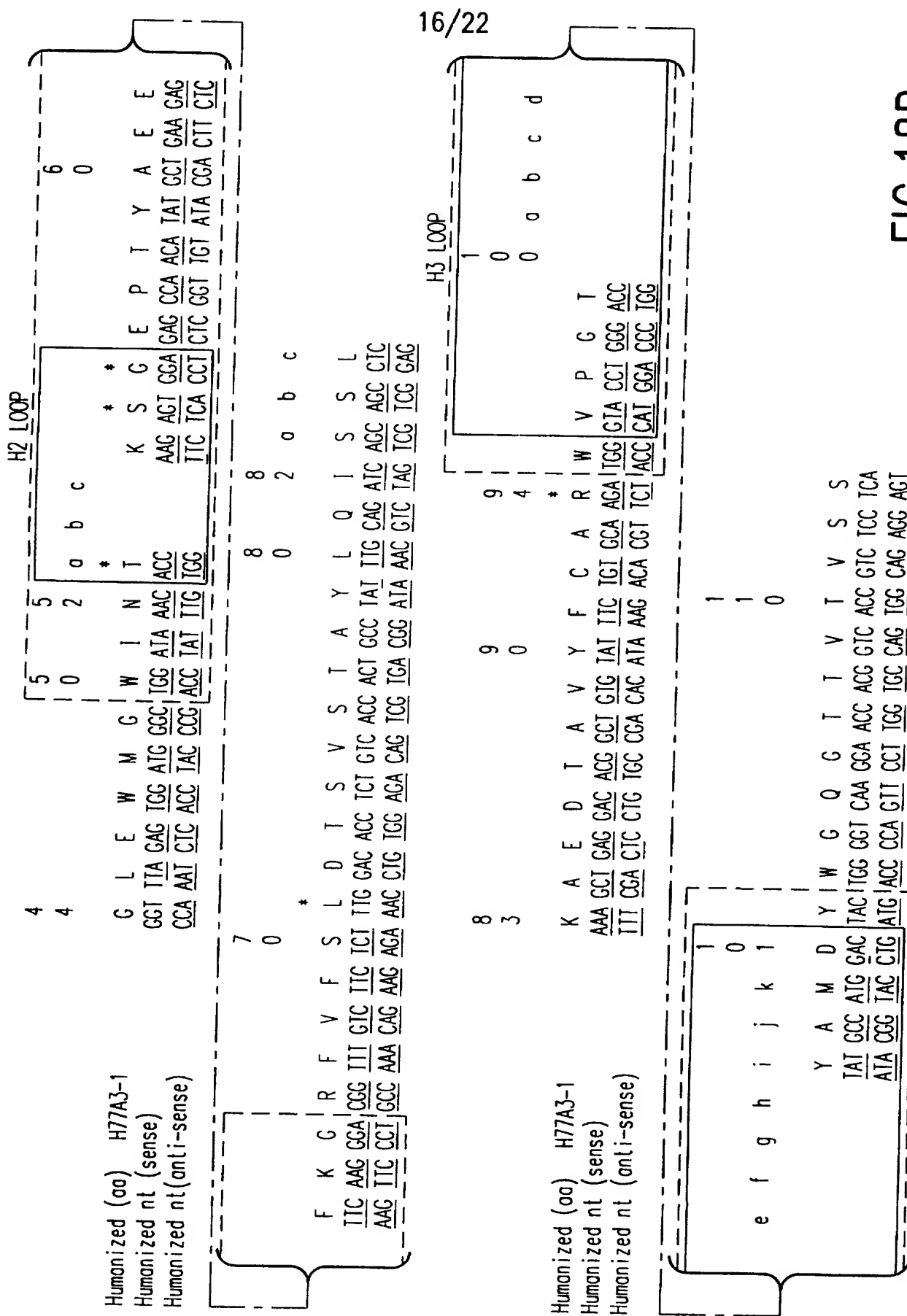
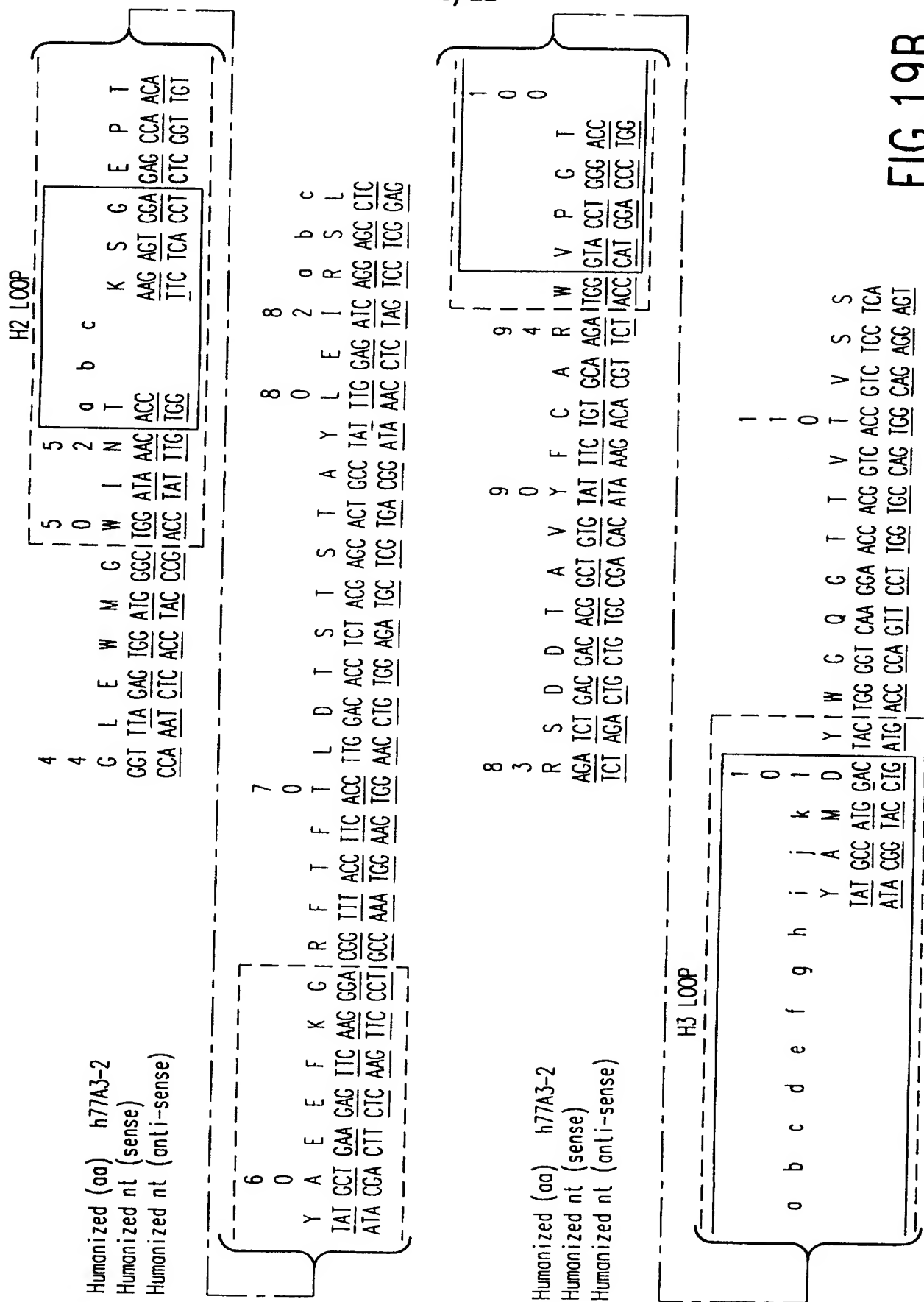


FIG.18B

Humanized (aa) h77A3-2
Humanized nt (sense)



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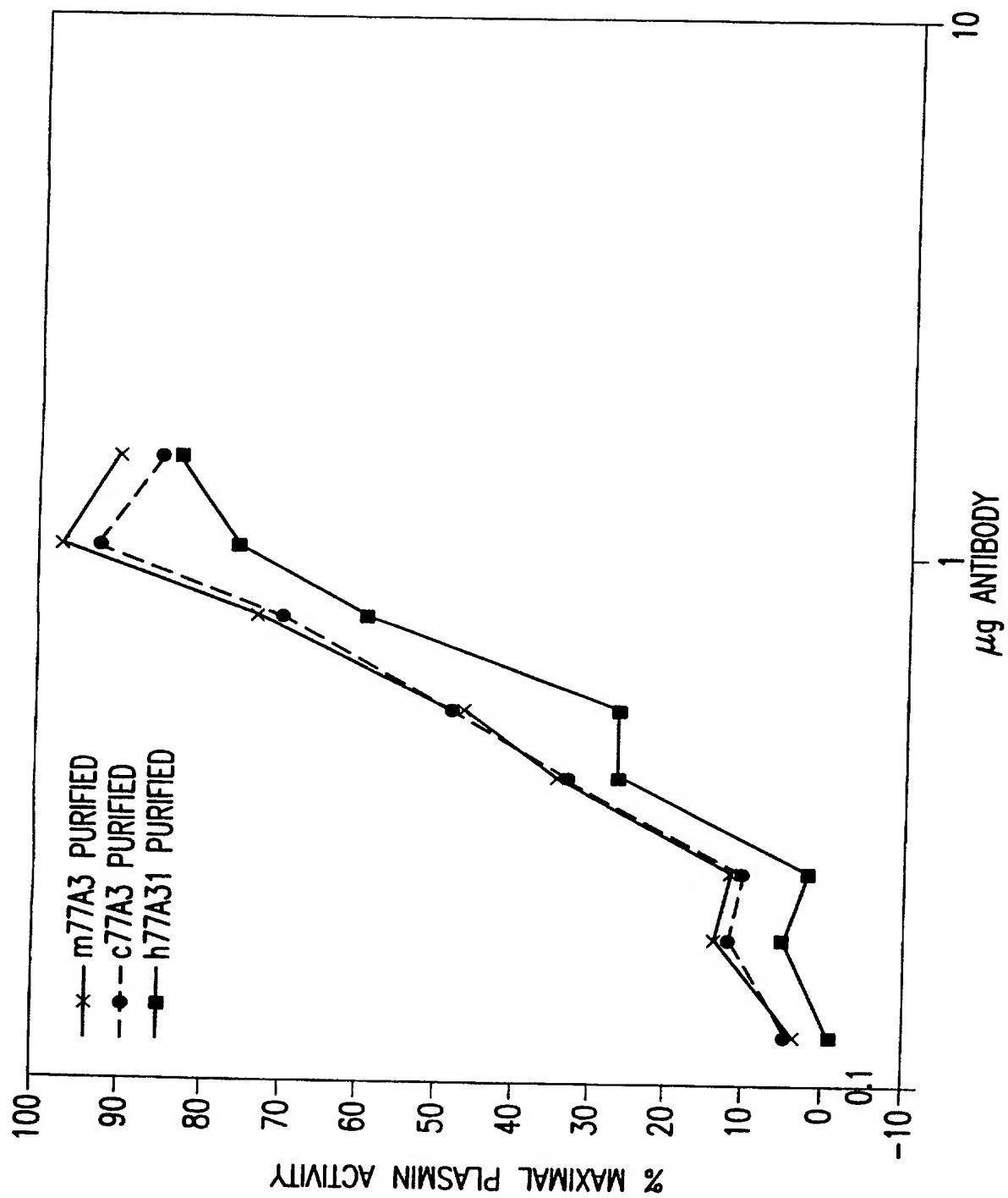


FIG.20

[illegible]

FIG. 21

[illegible]

h77A3-1
h77A3-2
m77A3
m49C9
m70B11
humanized consensus
murine consensus
77A3/49C9 consensus
all

[illegible]

h77A3-1
h77A3-2
m77A3
m49C9
m70B11
humanized consensus
murine consensus
77A3/49C9 consensus
all

FIG. 22A

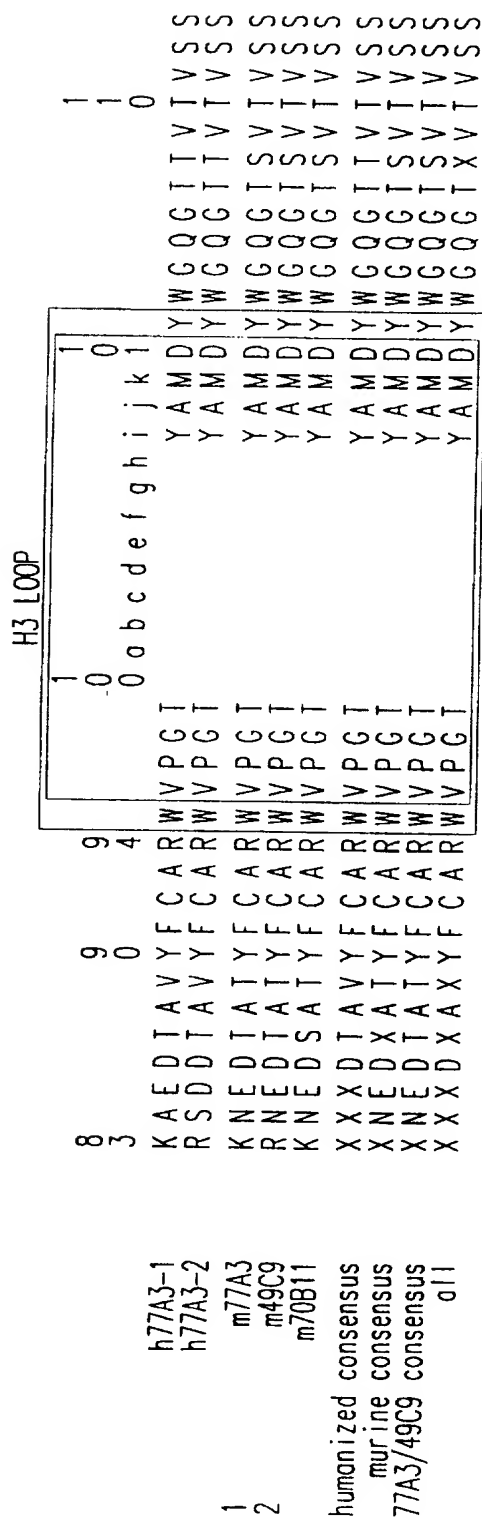


FIG. 22B